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# The Journal of Laboratory and Clinical Medicine

## Clinical and Experimental

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# REFLEX SHORTENING OF THE ESOPHAGUS IN THE EXPERIMENTAL ANIMAL WITH THE PRODUCTION OF ESOPHAGEAL HIATUS HERNIA

F. L. Dey, M.D., N. C. Gilbert, M.D., Ruth Trump, B.S., and R. C. Roskelley, M.D.
Chicago, Ill.

THERE are many reasons for the conclusion that esophageal hiatus hernia is an acquired condition, occurring most frequently in the fourth decade of life and later when the tissues about the hiatal orifice have become more relaxed. In a large part of the cases, an increase in intra-abdominal pressure may be assumed to be the determining cause.

But cases in which the hernia cannot be ascribed to an increase in intra-abdominal pressure do occur. These follow a pattern suggesting that some neurogenic factor is valent. Von Bergmann and Goldner,[1] and, later, Hurst[2] suggested that in many cases the occurrence of hiatus hernia might be due to traction of the stomach upward, due to a shortening of the esophagus in response to some vagal stimulation. Von Bergmann and Goldner quoted experiments by Kuckuck, showing that electrical stimulation of the vagus trunk caused a longitudinal contraction of the esophagus in the rabbit, which was capable of pulling the stomach up through the hiatal opening in the diaphragm.

This work was repeated in experiments upon the dog,[3] using Michel clips fastened to the stomach wall and the lower part of the esophagus to indicate their position in the roentgen plate. After control plates were made, the vagus trunk was stimulated electrically and pictures were taken during the stimulation. The esophagus was shown to be shortened by the stimulation of the vagus, and the stomach was pulled up to the diaphragm. These experiments were performed with the chest cavity closed, and the stomach was not pulled up through the hiatal orifice. Later, with the chest cavity opened, the cardiac end of the stomach was pulled up through the hiatal ring.

The present work was undertaken in order to determine whether or not vago-vagal reflexes could be elicited from mechanical stimulation of the peritoneum and viscera of the upper abdomen, which would reflexly shorten the esophagus and produce an esophageal hiatus hernia.

Dogs weighing between 7 and 10 kilograms were utilized in this study. The dogs were either decerebrated or else anesthetized by the method of Grehant,

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which, in our experience, is less prone to inhibit viscero-visceral reflexes than are some other anesthesias. A mechanical respirator was inserted in the trachea, and the vagi were isolated. With the dog on its right side, the left side of the chest was opened by resecting the ninth and tenth ribs, and a recording lever was attached to the esophagus, as shown in Fig. 1. A standard kymograph was utilized for recording deviations of the lever caused by shortening or lengthening of the esophagus. At the beginning of each experiment and at intervals throughout the experiment, control readings were made by stimulating the left vagus nerve in the neck by means of a Harvard inductorium.

A reflex shortening of the esophagus by means of various stimuli was demonstrated almost invariably from stimulation of one source or another in the upper abdomen. The ease with which these reflexes occurred varied inversely with the degree of depression caused by the decerebration or the anesthesia and also with the absence of shock due to insufficient anesthesia.

Mechanical stimulation of the peritoneum by scratching with a pointed instrument caused a reflex shortening of the esophagus in many of the animals. The effect of such stimulation is shown in Fig. 2. In this figure, as elsewhere, contraction or pulling up of the esophagus is shown by the upstroke, and relaxation or lengthening of the esophagus is shown by the downstroke. This reflex from irritation of the peritoneum was obtained in only some of the animals, but, in those in which it did occur, it was readily reproducible.

Reflex contraction of the esophagus was most readily obtained by any manipulation of the liver. The mere insertion of a finger between the leaves of the liver, such as in locating the gall bladder, was usually a sufficient stimulus. That the reflex was not due to peritoneal irritation is indicated by its occurrence in animals in which a reflex was not observed by mechanical stimulation of the peritoneum elsewhere.

Traction upon the right lobe of the liver also caused a reflex contraction of the esophagus independent of peritoneal irritation (Fig. 3). In applying traction to the liver, the diaphragm was pulled down, and with it, the esophagus. The initial downstroke in Fig. 3 is due to the traction of the esophagus downward by the initial traction upon the liver, and the upstroke is caused by the resulting reflex contraction of the esophagus. If very gentle traction upon the liver was made, so as not to disturb the position of the diaphragm, a similar shortening of the esophagus occurred, but with no downstroke (Fig. 4).

A reflex shortening of the esophagus also occurred if the muscle fibers of a small area of the stomach were stretched manually (Fig. 5). The demonstration of a reflex resulting from the manual stretching of a small area of stomach wall obviated the introduction of variables resulting from the distention of the entire stomach.

Reflex shortening of the esophagus also occurred as a result of traction made upon the gall bladder or of overdistention of the gall bladder with normal saline solution, or from distention of the cystic duct by passing a fine artery snap into the cystic duct and spreading it (Fig. 6).

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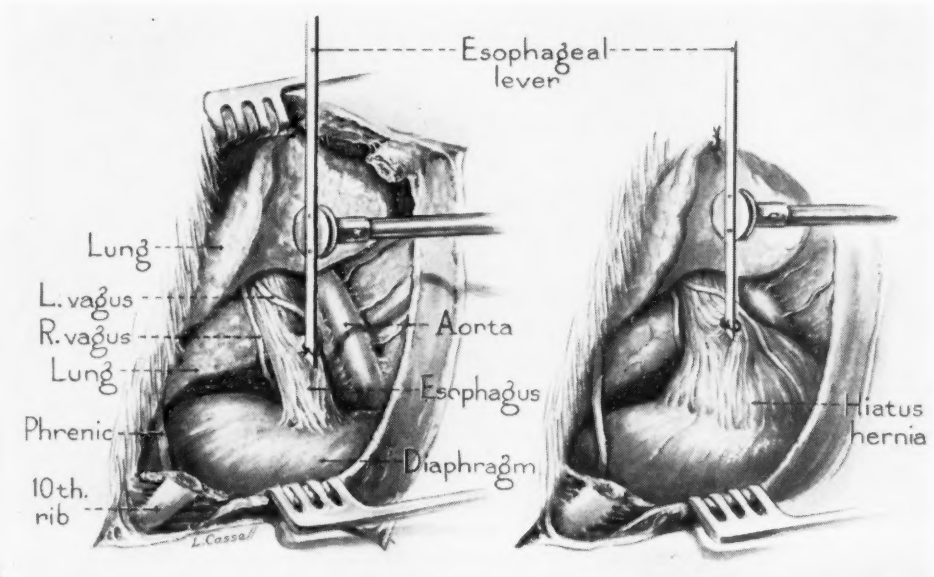


Fig. 1.—Drawings showing arrangement of apparatus as set up to record shortening or lengthening of the esophagus. The right-hand figure shows the effect of shortening the esophagus. The cardiac end of the stomach is pulled up to the hiatal orifice.

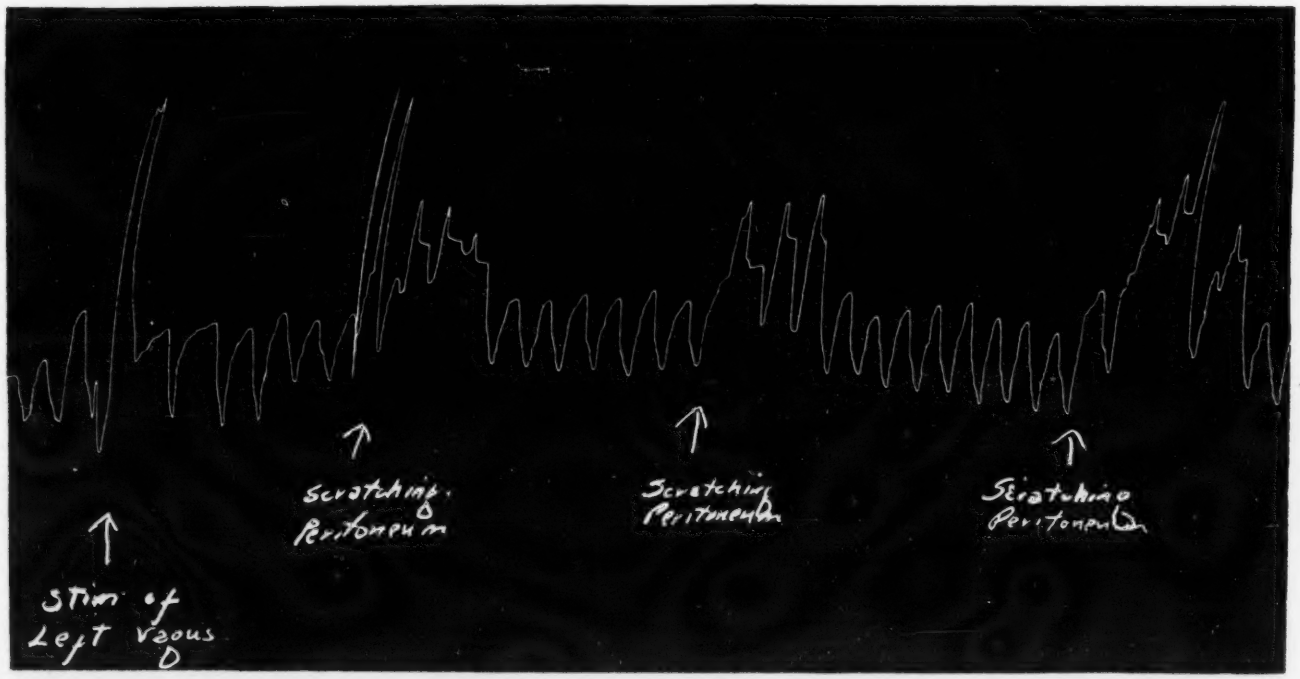


Fig. 2.—Upstroke shows shortening of the vagus due to electrical stimulation, followed by several incidents of reflex shortening of the esophagus after scratching the peritoneum with pointed instrument.

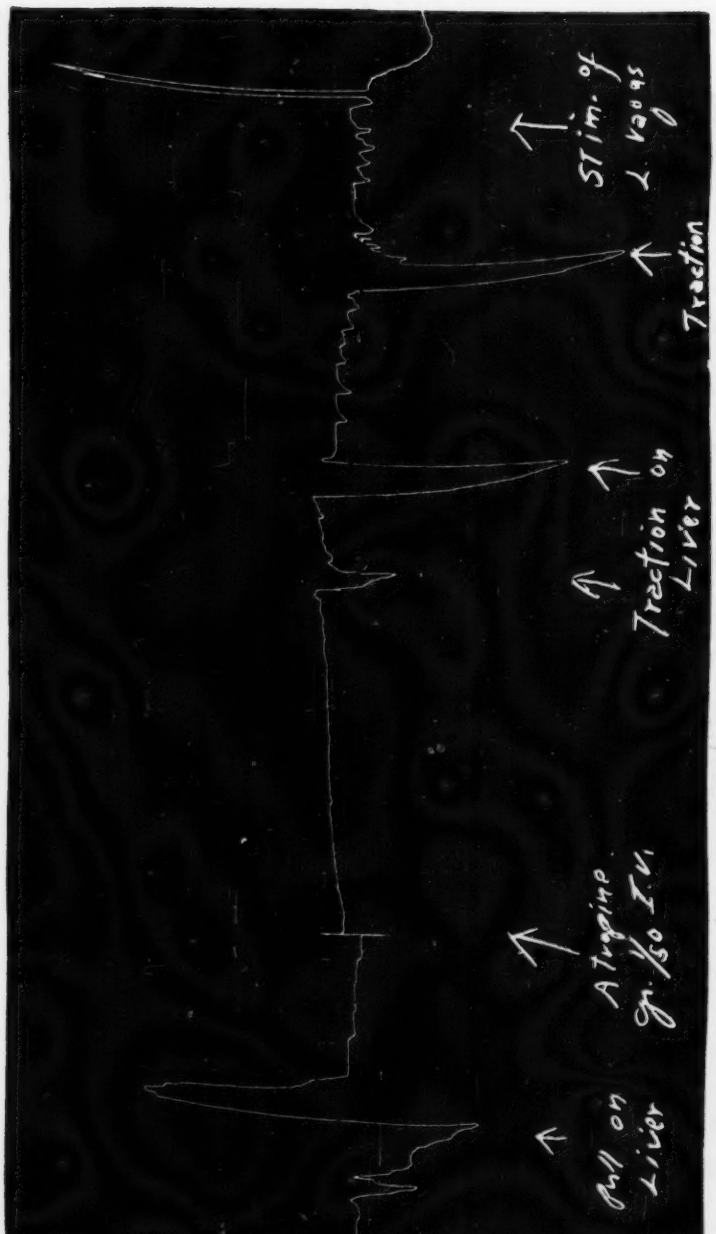


Fig. 3.—Effect of traction upon the liver; the initial downstroke is due to traction and lengthening of the esophagus, followed by an upstroke due to reflex shortening of the esophagus. Effect is abolished after atropine. Final upstroke is due to electrical stimulation of the left vagus.

In a few experiments, stimulation of the colon or of the urinary bladder by manipulation, traction, or overdistention failed to elicit a response, as did irritation of the rectum with croton oil or ether or by stretching of the sphincter muscle.

All of the reflexes shown in this work were abolished by vagal section or by atropine. Electrical stimulation of the central end of the cut vagus caused a shortening of the esophagus. This effect was not abolished by atropine, a finding to be reported upon later.

With the chest open, a very moderate increase in intra-abdominal pressure resulted in a protrusion of the cardiac end of the stomach into the chest cavity through the hiatal orifice. Shortening of the esophagus due to electrical stimulation of the vagus nerve in the neck, or to strong or repeated reflex stimulation, produced a similar herniation of the cardia.

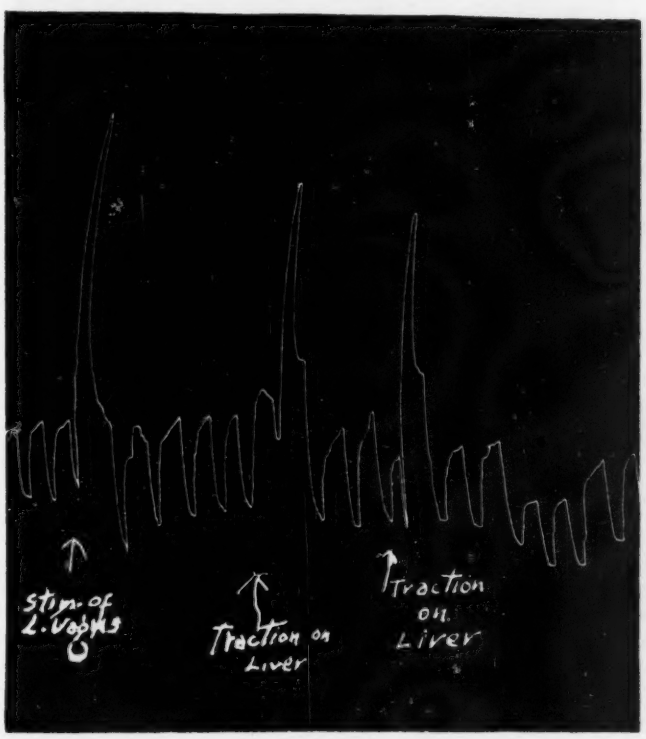


Fig. 4.—Reflex shortening of the esophagus following traction exerted so gently as not to pull on the diaphragm and esophagus.

It is recognized that the tissues comprising the hiatal ring in the dog are probably more lax than in man. In spite of this, we did not produce herniation in the dog with the chest closed and with the visceral pleura serving as a tamponade. That the muscles of the esophagus of the dog are entirely of the striated variety, instead of their distribution being confined only to the lower one-half or one-third of the esophagus as in man, should not make any difference in the interpretation of the results.

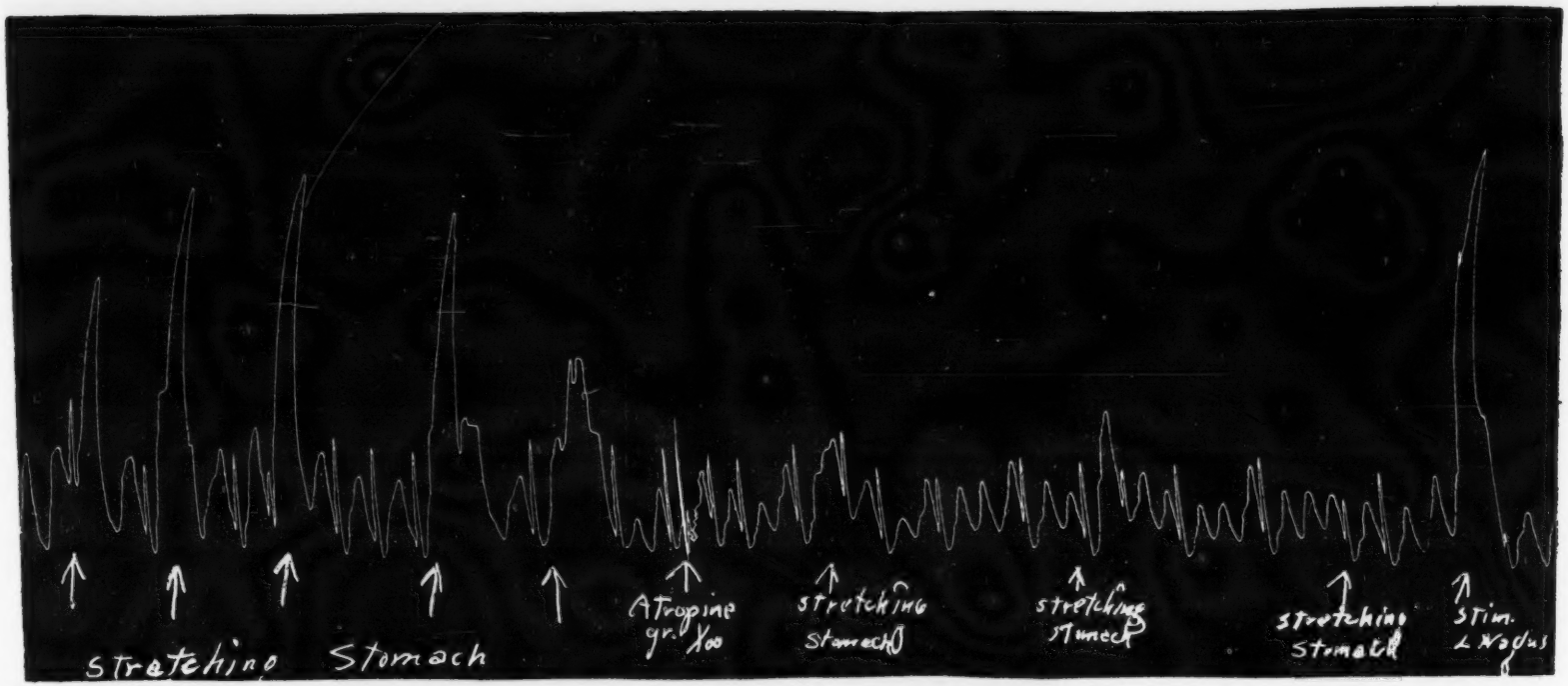


Fig. 5.—Effect of manually stretching small areas of the stomach wall. Reflex is abolished by atropine.

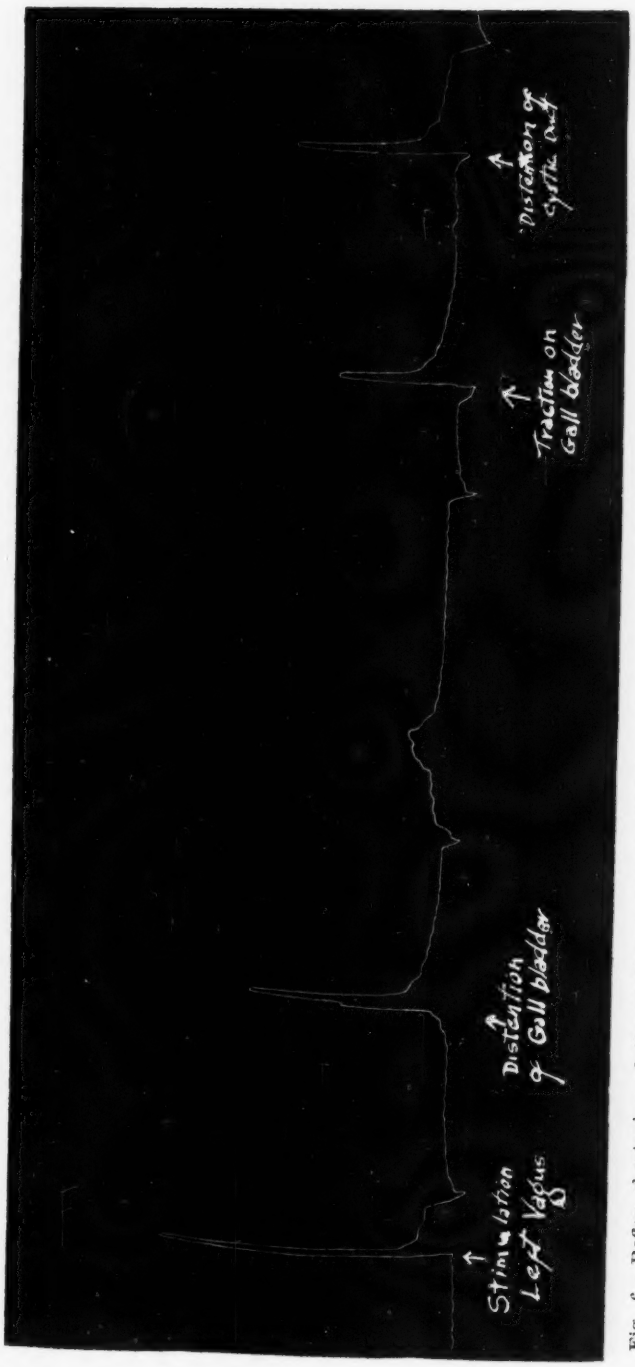


Fig. 6.—Reflex shortening of the esophagus due to distention of the gall bladder with normal saline, traction upon the gall bladder, and distention of the cystic duct.

## CONCLUSION

Shortening of the esophagus with the production of a hiatus hernia due to traction upon the stomach in the dog results from electrical stimulation of the vagus nerve or reflexly from stimuli having their origin in the viscera of the upper abdominal cavity.

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# A COMPARISON BETWEEN THE VALUES FOR PLASMA OR SERUM PROTEIN AS OBTAINED BY THE SPECIFIC GRAVITY AND THE MICRO-KJELDAHL METHODS

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MANY attempts have been made to perfect some simple and rapid method for the estimation of serum protein. The determination of nitrogen by the Kjeldahl procedure offers the most accurate means, but since it is time consuming and dependent on special equipment and requires a certain amount of skill, it has not proved adaptable in many instances where protein determinations would have been of great value in the physiologic assessment of subjects. In 1926, Barbour and Hamilton[1] described an accurate method for determining the specific gravity of serum or plasma. Moore and Van Slyke,[2] in 1930, found a high degree of correlation between the specific gravity of plasma and its protein content, and they expressed the relation between them by the formula:

$$\text{Total protein} = 343\ (\text{specific gravity} - 1.007)$$

Experience has shown that certain precautions are necessary for accuracy in determining specific gravity. In modifications of Barbour and Hamilton's method in which mixtures of xylene and bromobenzene are used as the standard medium, care must be taken to prevent evaporation of these volatile substances. In addition, the density of the standard solution of potassium sulfate or copper sulfate must be carefully checked. In the method described by Kagan,[3, 4] accurate results can be obtained if sufficient attention is given to maintaining drops of serum of uniform size and if the temperature is carefully controlled, but accuracy may be reduced if these factors are ignored.

Recently, Phillips and associates[5] described the use of copper sulfate solutions of different known densities in which a drop of serum or plasma either rises or falls, depending on its specific gravity. This method seemed to be particularly well adapted for use in field laboratories or on shipboard since no precision instruments are required. Uniformity in size of the drop is apparently unimportant, and no temperature correction is necessary because the coefficient of expansion of copper sulfate solutions approximates that of blood and plasma. Here again it is essential to use the utmost care in the preparation of the copper sulfate solutions of known specific gravity.

When plasma is used, it is necessary to apply a correction factor for the anticoagulant in the calculation of protein from the specific gravity. Experience in this laboratory has led us to the belief that many plasma specific gravity results are false because of inaccurate measurements of the volume of blood when taken in a syringe. For this reason it is preferable to use serum instead of

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plasma in order to eliminate slightly inconstant anticoagulant concentrations which make it impossible to apply an accurate correction figure.

Provided that all precautions are taken to ensure precise specific gravity determinations, the question next arises as to whether or not there is always a close correlation between the specific gravity of serum and its protein content. In 1942, Looney[6] studied the relation between the specific gravity of serum and its protein concentration in a group of patients with schizophrenia and also in a group of normal subjects. He found that the correlation between the two was too low to permit the use of specific gravity determinations for accurate serum protein estimations. Similar observations were made by Zozaya[7, 8] and by Moon and associates.[9] On the other hand, Atchley and co-workers[10] have stated that in their experience the copper sulfate method of Phillips and associates[5] for determining the specific gravity of plasma proved to be a simple and accurate method for the estimation of plasma proteins.

Several hundred protein determinations have been made in this laboratory since August, 1943, both by the micro-Kjeldahl method and by the copper sulfate specific gravity method of Phillips and associates.[5] One hundred twenty-eight of these comparisons have been selected for presentation here, because it was felt that the technique used in this group was the most free from criticism. Serum was used in all of these cases to eliminate any possible changes in specific gravity which might be caused by variations in oxalate concentration. The micro-Kjeldahl determinations were done by digestion with a mixture of concentrated sulfuric acid, copper sulfate, and potassium sulfate, with the addition of potassium persulfate as an oxidizing agent. In ninety-five of the reported cases, distillation was done with a simple condenser, and the distillates were nesslerized and read in a Klett-Summerson photoelectric colorimeter. Thirty-three of the determinations were distilled with a Keys closed ammonia still, and the distillates were titrated using a Rehburg burette. Since the results of the two methods showed no significant differences, they are considered together. Nonprotein nitrogen determinations were made by the Folin-Wu method.[11]

For the specific gravity determinations by the method of Phillips and associates, the copper sulfate solutions used were prepared according to their directions, and the final standards were checked for accuracy by pycnometric determinations for the density of the actual solutions used. The serum total protein was calculated from the density by the formula:

$$\text{Total protein} = 343\ (\text{specific gravity} - 1.007)$$

An analysis of these 128 comparisons is shown in Figs. 1 and 2. In Fig. 1, the differences found between the total proteins by the two methods are divided into groups covering 0.2 Gm. of protein per 100 c.c. of serum. The cases in which closest agreement between the two methods was found are shown in the center of the chart, with those in which the copper sulfate method gave lower results at the left of the center, and those in which the figures obtained from the copper sulfate method were higher at the right. It can be seen that in only forty-five instances, or 35 per cent, the variations between the results obtained by the two methods did not exceed ±0.2 Gm. of protein per 100 c.c. of serum.

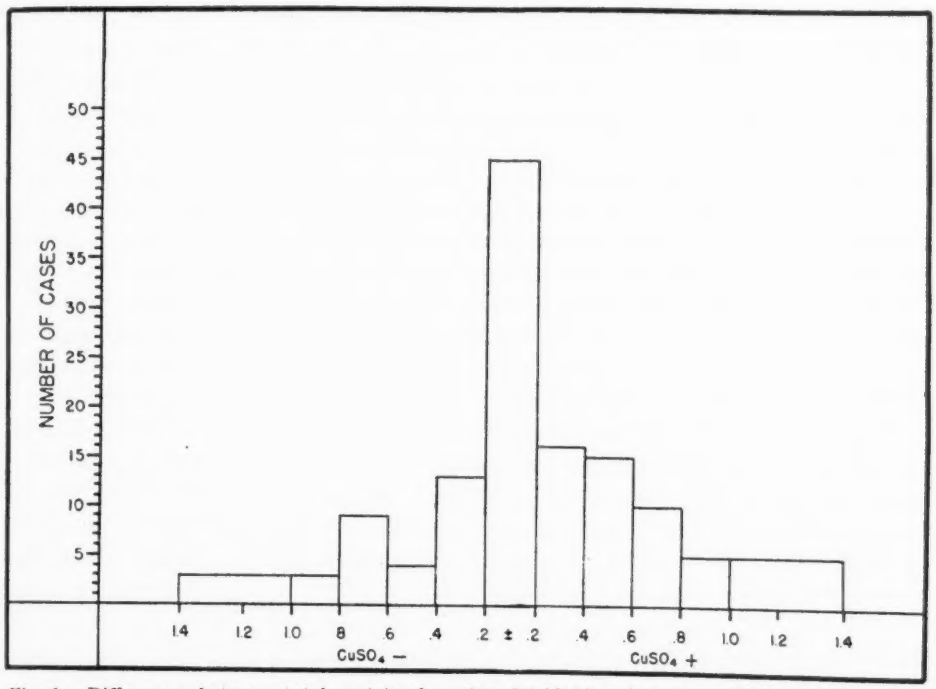


Fig. 1.—Differences between total proteins by micro-Kjeldahl and copper sulfate specific gravity methods.

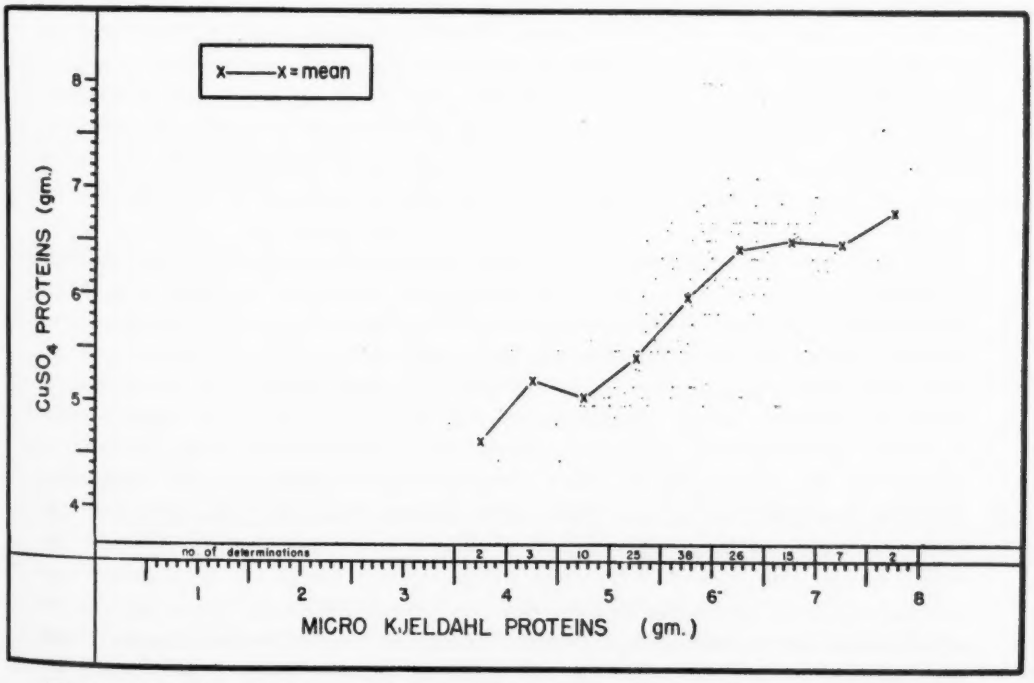


Fig. 2.—Correlation of the serum total proteins by copper sulfate specific gravity method with those by the micro-Kjeldahl method.

Duplicate micro-Kjeldahl results may be regarded as checks when the variations fall within this range, so that the two methods can be said to agree in forty-five of the 128 cases. In Fig. 1 are shown variations up to ±1.4 Gm. of protein per 100 c.c. of serum, with the larger discrepancies occurring in decreasing numbers of instances.

In Fig. 2 is demonstrated the graphical correlation between the serum total protein as determined by the micro-Kjeldahl method and the value obtained by calculation from the specific gravity of serum. It can be seen that the results by the copper sulfate method are scattered over a fairly wide range when compared with the micro-Kjeldahl determinations on the same samples of serum. To plot the line x—x in Fig. 2, the micro-Kjeldahl results were divided into groups covering 0.5 Gm. of protein per 100 c.c. of serum, and the mean copper sulfate value for the sera falling within each one of these micro-Kjeldahl groups was calculated. It can be seen that between protein values of 4.75 and 6.25 Gm. per 100 c.c. of serum the line x—x follows a straight line very closely and that below and above these points there appears to be less correlation. However, the greatest differences from the mean occur in this group of patients and a better evaluation of correlation may be obtained by calculating the coefficient of correlation by the method described by Mainland.[12, pp. 249-252] The coefficient of correlation for the whole series of 128 cases was found to be 0.74. When the cases were divided into groups according to low, normal, and high serum protein content by the micro-Kjeldahl method, the following values for the coefficient of correlation were obtained: In forty cases having total proteins between 3.45 and 5.50 Gm. per 100 c.c. of serum, the correlation coefficient for the two methods was 0.51; in seventy-nine with total protein content ranging from 5.50 to 7.00 Gm. per 100 c.c. of serum, the correlation coefficient was 0.45; and in nine in which the total protein was higher than 7.00 Gm. per 100 c.c. of serum, the coefficient of correlation was found to be 0.52. Apparently correlation between the two methods did not vary greatly with the total protein content of the serum. It is to be pointed out that the last group of nine cases is too small to be of statistical value.

Inspection of the albumin to globulin ratio in 125 of the 128 cases studied suggests that either an elevated globulin or a decreased albumin may have some effect on the serum specific gravity. The cases were divided into groups according to their albumin and globulin content as determined by the micro-Kjeldahl method. An albumin content above 3.0 Gm. and a globulin content below 3.0 Gm. per 100 c.c. of serum were regarded as normal. Within each of these groups, the total protein values as determined by the two methods were examined for differences not exceeding ±0.3 Gm. per 100 c.c. of serum, which is the degree of accuracy found by Phillips and associates[5] for the copper sulfate method. In 45 per cent of the cases in which the albumin concentration was within normal limits, the total protein as calculated from the specific gravity did not vary significantly from the results obtained by the micro-Kjeldahl determination. When the globulin content was normal, the results obtained by the two methods agreed closely in 48 per cent of the cases. These figures are similar to those found for the entire series of 125

cases in which the albumin and globulin were determined. In 44 per cent of the 125 cases, there were no significant variations between the total protein values found by the two methods.

In the group of sera having albumin concentrations below 3.0 Gm. per 100 c.c., the specific gravity method agreed with the micro-Kjeldahl in only 38 per cent of the cases. In all but one of the other cases of this group, the total protein calculated from the specific gravity was higher than that found by the micro-Kjeldahl determinations. When the globulin content was above 3.0 Gm. per 100 c.c. of serum, close agreement between the two methods was found in only 31 per cent of the cases. The remaining cases of this group were found to be almost equally divided between those with increased specific gravity and those in which the specific gravity was decreased.

Determinations of other blood constituents were not followed carefully enough in most of the cases in this group of 128 determinations to shed any light on the reasons for the observed variations between the Kjeldahl and specific gravity methods.

Most of the patients were hospitalized because of burns. A few of the determinations were done soon after the injury, but more of them were on patients who had been in the hospital for long periods of time and were being followed because of interest in wound healing and nutrition. In the hope of finding some biochemical conditions which might have had some consistent effect on the specific gravity of serum or plasma, the records of a larger group of patients were studied with particular reference to low serum electrolyte content, the presence of shock, and hemoconcentration. In most of these additional cases, plasma had been used instead of serum for the protein determinations.

In nine blood samples showing a low sodium content, the total protein as calculated from the specific gravity method did not differ from that obtained by the micro-Kjeldahl by more than ±0.2 Gm. per 100 c.c. of plasma in three instances. Two comparisons showed a higher protein as estimated from the specific gravity, the difference being +0.26 and +0.89 Gm. per 100 c.c. of plasma, respectively. In four instances, the specific gravity method gave lower values, and the differences were –0.21, –0.36, –0.45, and –1.00 Gm. of protein per 100 c.c. of plasma.

Serum chloride values lower than 99 meq./l were observed in seven samples of serum in which the protein was determined by both methods. The data are given in Table I. No data were available in which extremely low chloride values were found. A third sample of serum is included for Case 3 to show that there was still a wide variation between the protein values obtained by the two methods even though the serum chloride had become normal.

The plasma proteins of fifteen patients in shock were compared by both protein methods. In seven of the fifteen, the protein values by the two methods agreed with variations not greater than ±0.2 Gm. per 100 c.c. of plasma. Four were found to have proteins higher by more than 1.00 Gm. per 100 c.c. of plasma by the specific gravity method than by the micro-Kjeldahl procedure. In the other four cases, the specific gravity method gave lower values, the differences varying from –0.37 to –1.56 Gm. of total protein per 100 c.c. of plasma.

TABLE I

| CASE | DATE | TOTAL PROTEIN BY MICRO-KJELDAHL METHOD (GM. PER 100 C.C.) | TOTAL PROTEIN BY $CuSO_4$ METHOD (GM. PER 100 C.C.) | VARIATION OF $CuSO_4$ FROM KJELDAHL METHOD | CHLORIDE (MEQ./1) |
|---|---|---|---|---|---|
| 1 | 10/27/44 | 6.54 | 6.17 | −0.37 | 96.1 |
| | 10/28/44 | 5.89 | 6.93 | +1.04 | 96.2 |
| | 10/29/44 | 6.34 | 8.00 | +1.66 | 95.3 |
| 2 | 11/14/44 | 5.56 | 5.56 | 0 | 96.7 |
| | 11/15/44 | 5.64 | 5.49 | −0.15 | 88.9 |
| 3 | 9/28/45 | 4.32 | 3.02 | −1.30 | 88.5 |
| | 9/29/45 | 5.41 | 3.46 | −1.95 | 94.5 |
| | 10/11/45 | 6.67 | 5.66 | −1.01 | 104.6 |

Both methods for determining the total protein of plasma or serum were used in thirty-six patients in whom hemoconcentration was observed. As shown in Table II, the variations between values obtained by the two methods were distributed fairly evenly over a wide range, and it was impossible from this group of cases to draw any conclusions as to the possible effect of hemoconcentration on the specific gravity of plasma or serum.

TABLE II. PATIENTS WITH HEMOCONCENTRATION

| DIFFERENCE BETWEEN TOTAL PROTEIN BY MICRO-KJELDAHL AND $CuSO_4$ METHODS (GM. PER 100 C.C. PLASMA) | NUMBER OF CASES |
|---|---|
| $CuSO_4$ ±0 to 0.20 | 5 |
| $CuSO_4$ +0.21 to 0.40 | 6 |
| $CuSO_4$ −0.21 to 0.40 | 4 |
| $CuSO_4$ +0.41 to 0.60 | 3 |
| $CuSO_4$ −0.41 to 0.60 | 3 |
| $CuSO_4$ +0.61 to 0.80 | 2 |
| $CuSO_4$ −0.61 to 0.80 | 3 |
| $CuSO_4$ +0.81 to 1.00 | 2 |
| $CuSO_4$ −0.81 to 1.00 | 3 |
| $CuSO_4$ +more than 1.00 | 5 |
| $CuSO_4$ −more than 1.00 | 0 |

If a larger number of cases were studied with analysis for other blood constituents and with more careful observations of the patient's condition, it might be possible to determine under what conditions it is safe to rely on the specific gravity as a means of calculating the serum protein. However, from our observations of many random cases in which the protein content was estimated by both the micro-Kjeldahl and by the copper sulfate specific gravity method, it was impossible to draw any conclusions regarding the causes for variations between the two methods. It is obvious from Figs. 1 and 2, which present a series of determinations in which meticulous laboratory technique was used, that it is unsafe to place too much confidence in serum protein values as estimated from the specific gravity when accurate determinations of protein are required.

## SUMMARY

1. One hundred and twenty-eight cases are presented in which the serum protein concentration was determined by both the micro-Kjeldahl procedure and by the copper sulfate specific gravity method.

2. In fifty-five of the 128 cases, or 44 per cent, the differences between the total protein values by the two methods did not exceed 0.3 Gm. per 100 c.c. of serum. The percentage of cases showing such agreement was slightly lower when the albumin was below normal or the globulin above normal. When 0.2 Gm. per 100 c.c. is taken as the criterion for duplication, only 35 per cent of the cases showed such agreement.

3. No conclusions could be drawn as to the type of patient in whom the serum specific gravity is influenced by factors other than the protein content.

4. The correlation between the specific gravity of serum and its protein content was found to be too low to permit the use of specific gravity determinations for reliable estimation of serum total protein.

The authors wish to thank Miss Dorothy Keller and Miss Jeanette Maioli, for doing many of the nitrogen and specific gravity determinations.

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# STUDIES ON STREPTOMYCIN*

## I. Assay in Body Fluids

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With the Technical Assistance of

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VARIOUS methods of assaying antibiotics described in the literature are for the most part modifications of the cylinder-plate method of Abraham and associates[1] introduced as improvements on the original method. This is particularly true of penicillin. Foster and Woodruff[2] summarized and discussed the principles, merits, and disadvantages of different bacteriologic methods for the quantitative determination of penicillin and recommended a modification of the Oxford cup method for routine assays. According to Schmidt and Moyer,[3] agar depth, stock culture of the test organism, pH of the medium, and other factors influence results obtained when the cup method is used. A stock suspension of *Bacillus subtilis* spores was recommended in preference to *Staphylococcus aureus* for penicillin assay by Foster and Woodruff.[4] Similarly, *B. subtilis* was employed by these authors for streptothricin assay.[5] For convenience and economy, large Pyrex baking dishes were used by Epstein and co-workers,[6] while Beadle, Mitchell, and Bonner[7] found large, rectangular culture plates advantageous for speed and consistency in routine assay work.

Simplifications of the procedure for applying the assay material to the agar surface have been reported. Cholden[8] used a rubber stopper technique to make depressions in the agar to hold the assay liquid. Filter paper disks, instead of cups, were used by Sherwood, Falco, and de Beer,[9] Vincent and Vincent,[10] and Epstein and co-workers.[6]

Different streptomycin assay methods have been suggested and described. The turbidimetric method, the serial dilution procedure with *Escherichia coli* as the test organism, and the cylinder-plate technique using *B. subtilis* cells or spores were suggested by Waksman, Bugie, and Schatz.[11] Heilman[12] recommended a modification of Fleming's slide-cell technique for estimating the concentration of streptomycin in body fluids. This method was considered inferior to the cup-plate method for urine assay. To determine the degree of inactivation of streptomycin and streptothricin by cysteine, Denkelwater, Cook, and Teshler[13] employed the cup method for assay. Using as a test organism a strain of *Staph. aureus*, Stebbins and Robinson[14] found the cylinder-plate procedure satisfactory for the quantitative determination of streptomycin in the blood.

This investigation was conducted at Camp Detrick, Frederick, Md., from December, 1944, to October, 1945.

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*Streptomycin as referred to in this paper is streptomycin hydrochloride.

In the present report, a method is described for assaying streptomycin in body fluids, particularly in blood and urine. This method was developed for investigating the absorption, excretion, and activity of the drug in human beings and in laboratory animals, and it appears to be accurate and convenient for assaying numerous samples. The results of the procedure are based on the relationship between the diameter of the zone of inhibition and the amount of streptomycin diffusing into an agar plate seeded with a sensitive variant strain of *B. subtilis* Cohn *emend.* Prasmowski. Special assay agar was used.*

## EXPERIMENTAL METHODS

*Preparation of Spore Suspension.—*

*Strain:* A variant strain of *B. subtilis* Cohn *emend.* Prasmowski, designated in this laboratory as the I strain, was chosen for the standard assay organism. It was preferred to several other organisms for the following reasons: (1) It sporulated readily on media used for spore production. (2) The viability of the spores was maintained better under the conditions of storage. (3) The zones of streptomycin inhibition were clearer and more defined. (4) The inhibitory action of normal blood observed when using *B. subtilis* var. *viscosus* Chester, has not been observed to date with human, rabbit, guinea pig, mouse, and dog blood, whether normal or infected.

*Preparation of Inoculum for Spore Suspension:* A pure culture was obtained by streaking the organisms (previously subcultured several times in dextrose broth) on dextrose agar, incubating at 37° C. for twenty-four hours, and transferring several rough, granular colonies onto slants. The slants were incubated for forty-eight hours at 37° C., and from them a suspension was made and used for inoculating large dextrose agar slants in thirty-two ounce prescription bottles. These were incubated for forty-eight hours, and a 50 c.c. aqueous suspension of the organisms was made from each bottle for mass inoculation of the media to be used for spore production.

*Sporulation:* Five cubic centimeters of the suspension described were added aseptically to beef heart infusion dextrose agar† slants in thirty-two ounce prescription bottles, stoppered with cotton plugs. Each bottle was tilted several times to facilitate inoculation of the entire surface of the slant. The bottles were incubated at 37° C. for approximately ten days, during which time they were examined grossly for contamination and microscopically for degree of sporulation. It was observed that a grayish-white, dry appearance of the colony indicated sporulation.

*Now available as Difco E-2 Streptothricin Assay Agar, Difco Laboratories, Incorporated, Detroit, Mich.

†Beef heart infusion dextrose agar:

| | |
|---|---|
| Beef heart infusion (dehydrated) (Beef heart infusion from 500 Gm. beef heart) (Bacto tryptose, 1.0%) (Sodium chloride, 0.5%) | 2.5% |
| Bacto-peptone | 1.0% |
| Bacto-agar | 1.5% |
| Dextrose (chemically pure) | 1.0% |
| Distilled water (q.s. add.) | 1,000 c.c. |

Preliminary pH, 7.3; final pH, 7.0
Autoclave, 15 pounds per 20 minutes

*Removal and Washing of Spores:* After sporulation was almost complete, approximately 90 per cent, the growth was removed from the agar by adding approximately 5 Gm. of sterile glass beads and 10 c.c. of sterile 1.0 per cent phosphate buffer, approximately pH 7.0, and by rotating the bottle carefully from side to side. (By adding 5 Gm. each of monobasic and dibasic potassium phosphate to 1 L. of distilled water, a 1 per cent buffer of the desired pH can be obtained.) The resulting slurry and beads were then transferred into a sterile Erlenmeyer flask, and the bottle containing the slant was rinsed twice with 5 c.c. of sterile diluent. The suspension was shaken thoroughly and filtered through a sterile Buchner funnel containing sterile cheesecloth to remove the beads and bits of agar. The slurry was then dispensed into sterile centrifuge tubes, thoroughly mixed with a convenient quantity of the 1 per cent phosphate buffer, and centrifuged. This process of washing and centrifuging was repeated three times, and the washed spores were then transferred to a container and stored at 2° C. A sample was removed for microorganism count, after which part of the suspension was diluted to give the desired microorganism concentration (preferably eighty million per cubic centimeter) and stored at 2° C.

A spore suspension, prepared in the manner described, has now been used successfully for ten months. Thus, small laboratories can be spared the burden of producing spore suspensions, since a standardized product can be prepared in a central laboratory and dispensed as needed. Furthermore, recent experiments indicate that the spore suspension can be lyophilized with practically no loss in viability of the spores, facilitating ease of shipping.

*Assay Technique.—*

*Preparation of Seeded Plates:* For assaying, 200 c.c. of agar* were melted, cooled, and maintained at from 49 to 50° C. Prior to assay, the agar was seeded with *B. subtilis* spores (approximately 400,000 spores per cubic centimeter of agar) and maintained at 50° C. until all of the air bubbles emerged to the surface. Portions of 25 c.c. inoculated agar were transferred to large, flat-bottomed Petri plates (150 by 20 mm.), using a 30 c.c. glass syringe, equipped with an automatic syringe filler to facilitate rapid pouring. Seeded plates were allowed to cool and harden for ten minutes at room temperature. They were then dried uncovered at 50° C. for ten minutes to remove condensed moisture and allowed to return to room temperature for ten minutes with covers partly open.

It is essential that plating be performed on a level table to obtain a uniform depth of agar and that air bubbles disappear from the agar before it hardens. All timing should be carefully checked.

Plates prepared for assay can be stored at 2° C. for future use. However, there is a loss of accuracy proportional to length of storage time. Such plates must be redried at 50° C. and then allowed to attain room temperature before use.

*Addition of Assay Material:* Each plate selected for assay was superimposed over an area marked on a *level* table so that assay disk pads† could be placed in

*Difco E-2 Streptothricin Assay Agar.

†Schleicher and Schuell 740E one-half inch analytical filter pads were used for assay disks.

the same relative position on each consecutive plate (see Fig. 1). The material to be assayed was drawn into a 1 c.c. tuberculin syringe, graduated in hundredths, carrying a 26-gauge, 1-inch needle. The syringe was inverted and the air bubbles expelled, except in the assay of very dark fluids, where the retention of a few small air bubbles aided materially in establishing a sharp line of demarcation at the base of the plunger. Using forceps, an assay pad was carefully dropped onto the agar so that the *smooth* side of the pad was in contact with the agar surface. Precisely 0.15 c.c. of fluid was then *immediately* ejected from the syringe onto the pad. (In order to deliver 0.15 c.c. of assay material accurately, it is necessary that the barrel of the syringe be grasped between the thumb and forefinger of one hand and that the plunger be operated with a slight twirling motion with the thumb and forefinger of the other hand.) This

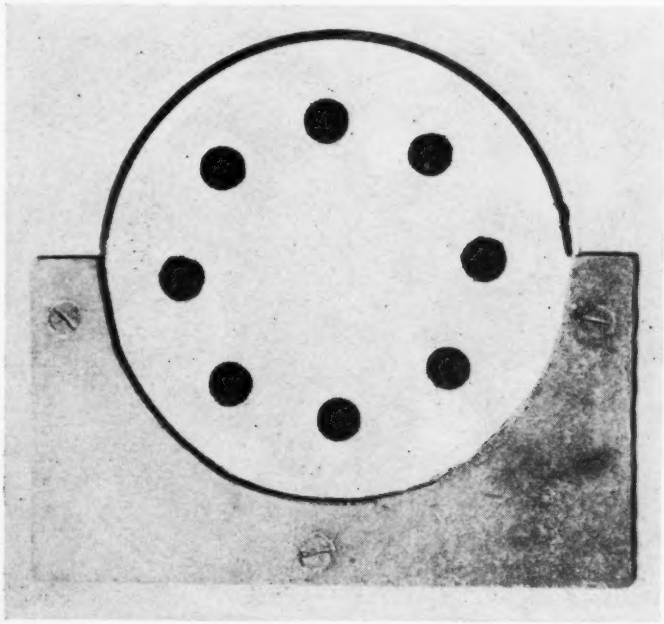

Fig. 1.—The pattern used for superimposing seeded agar plates in order to place assay pads on the same relative position on each plate.

procedure was carried out in quintuplet for all samples assayed, one pad on each of five plates. Using this procedure, samples of assay material sufficient to assay a series of five plates previously poured and prepared can be completed in from fifteen to twenty minutes. A team of two persons is recommended for efficient assay operation with this procedure. However, one individual can successfully complete the operation.

*Incubation and Reading of Assay Plates:* The assay plates were incubated for seventeen hours at from 28 to 30° C., removed from the incubator, and the diameter of the zone of inhibition of growth measured (see Fig. 2). (Although assays could be completed in eight hours, a seventeen-hour incubation period was adopted for convenience.) Readings were recorded to the nearest 0.1 mm.

Values should be within plus or minus 1.0 mm. of one another. For example, if five readings for any hypothetic value are 21.0, 21.6, 20.9, 21.4, and 22.7, the last value (22.7) should be discarded. The average reading of the diameters of the zone of inhibition for each standard was plotted on graph paper against units per cubic centimeter for the respective standards. The diameter of the inhibition zone for any unknown assay sample was compared with the standard curve to give results in units of streptomycin per cubic centimeter of fluid.

Any plate-counting apparatus can be used for reading the inhibition zones, provided it is equipped with a transparent graduated film ruled in 1 mm. areas. The diameter can also be read with a millimeter rule. However, it

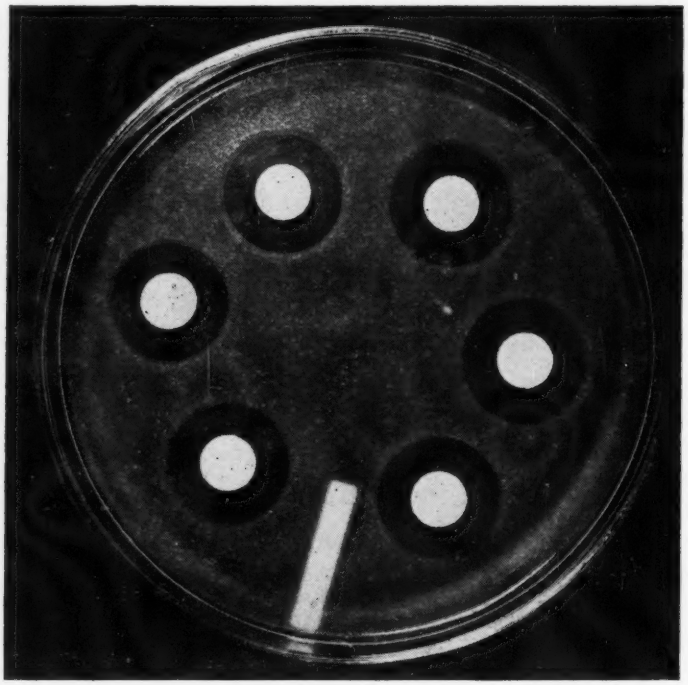

Fig. 2.—Illustrating the type of inhibition zone produced by streptomycin, using the pad method of assay.

was found convenient to use a positive exposed negative of 1 mm. graph paper on lithographic film.

*Preparation of Standard Curves:* Prior to treatment, a sample of body fluid, that is, blood or urine, was obtained for preparing a streptomycin standard curve. To aliquots of the selected fluid, varying concentrations of streptomycin were added and then assayed as previously described. The diameter of the resulting zone of inhibition was then plotted on graph paper against the corresponding unitage (see Figs. 3, 4, and 4*A*). For example, in following the blood level of streptomycin in a patient, a sample of blood was taken before the administration of the drug for constructing a standard curve on blood of that

particular person. Blood was collected in lithium oxalate (0.06 c.c. of a 3.3 per cent lithium oxalate solution per 1 c.c. of blood) and mixed. Then 0.9 c.c. of blood was accurately pipetted into sterile tubes, and to each of these 0.1 c.c. of streptomycin solutions of given variable unitage was added. (In pipetting blood for preparing standards, the outer surface of the pipette must be wiped to remove excess blood after it has been filled and before delivery to the test tube, and blood must be allowed to flow slowly from the pipette into the test tube from the 0 to the 0.9 mark.) A blank was used as a check to determine possible normal *B. subtilis* inhibiting properties of the blood. Water standards of streptomycin were prepared so that when 0.1 c.c. was added to 0.9 c.c. of blood or other liquid, concentration of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 units of

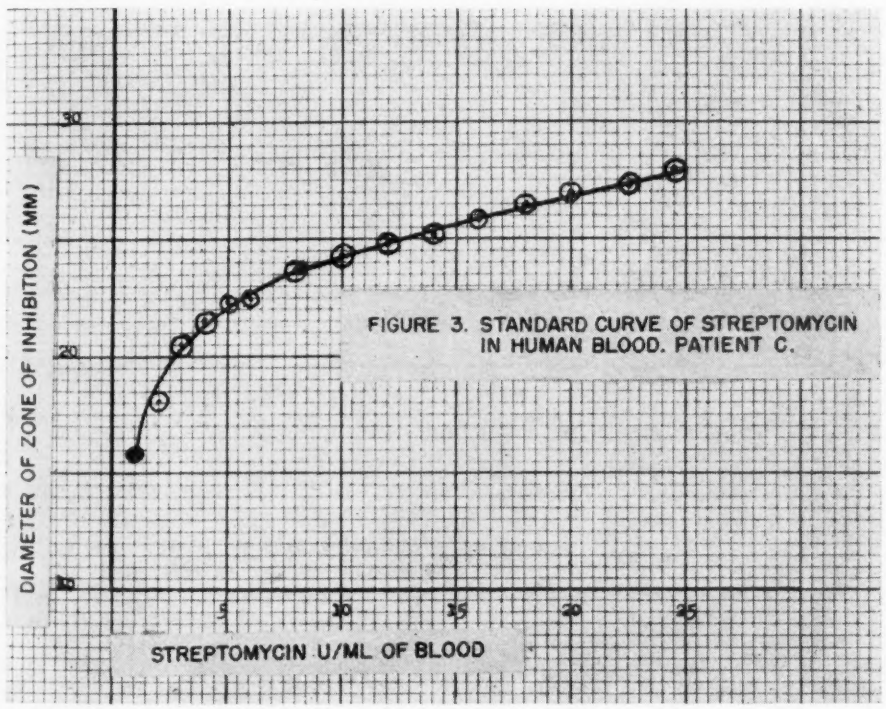


FIGURE 3. STANDARD CURVE OF STREPTOMYCIN IN HUMAN BLOOD. PATIENT C.

Fig. 3.

streptomycin per cubic centimeter resulted for the low unitage curve, and up through 30 units per cubic centimeter in steps of 2 units for the high unitage curve. (Standard solutions in water must be made so that the resulting unitages are 5, 10, 20, 30, 40, etc. Thus, when 0.1 c.c. of each standard is added to 0.9 c.c. of blood, the resulting unitage is decreased by one decimal.) Streptomycin standards from 0 through 6 units were placed on one series of five plates; standards from 8 through 18 and 20 through 30 were placed on two other series, respectively.

It is recommended that until experience is gained with the method a complete standard curve should be run from 0 to 30 units per cubic centimeter.

This will necessitate obtaining a 20 c.c. sample of blood from the patient before administration of streptomycin. From experience with correlating blood levels and dosages, one can soon learn to select that portion of the curve that will be required to determine blood levels from a given dose. This will permit the use of a smaller blood sample for making the standard curve. Only a 1 c.c. sample of blood is required for determining the blood level of a patient in treatment.

Fig. 4.

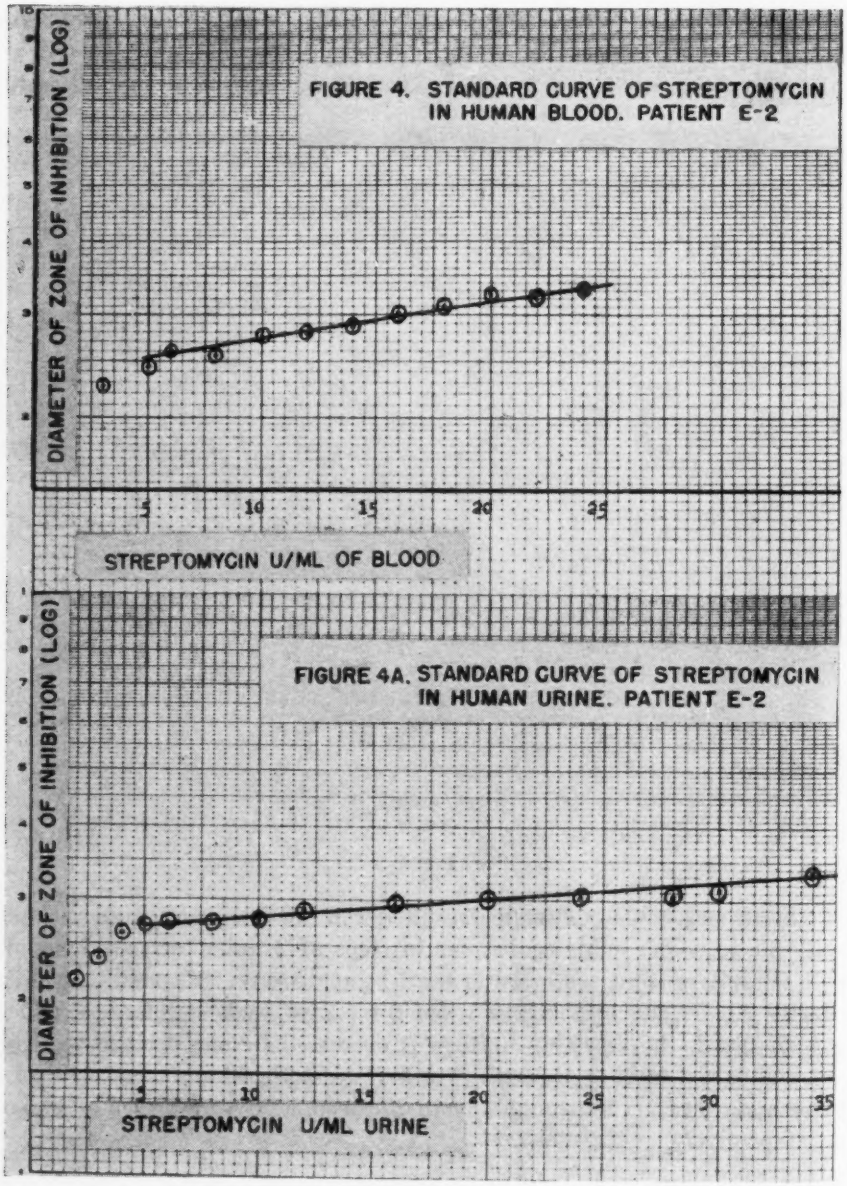
FIGURE 4. STANDARD CURVE OF STREPTOMYCIN IN HUMAN BLOOD. PATIENT E-2
DIAMETER OF ZONE OF INHIBITION (LOG)
STREPTOMYCIN U/ML OF BLOOD
FIGURE 4A. STANDARD CURVE OF STREPTOMYCIN IN HUMAN URINE. PATIENT E-2
DIAMETER OF ZONE OF INHIBITION (LOG)
STREPTOMYCIN U/ML URINE

Fig. 4A.

TABLE I. VARIATIONS IN INDIVIDUAL STANDARD CURVES

| STREPTOMYCIN ADDED (UNITS PER C.C.) | DIAMETER OF ZONE OF INHIBITION (MM.) | | | |
|---|---|---|---|---|
| | PATIENT D | PATIENT E | PATIENT F | PATIENT C |
| 0 | 0 | 0 | 0 | 0 |
| 0.5 | 15.8 | 16.0 | 16.4 | 0 |
| 1 | 18.4 | 20.0 | 18.2 | 15.8 |
| 2 | 20.4 | 21.1 | 22.5 | 18.1 |
| 3 | 21.5 | 21.7 | 24.3 | 20.5 |
| 4 | 21.8 | 22.4 | ---- | 21.6 |
| 5 | 22.0 | 22.6 | 22.5 | 22.3 |
| 6 | ---- | 23.4 | 26.2 | 22.4 |
| 8 | 22.4 | 24.9 | 27.4 | 23.6 |
| 10 | 22.6 | 25.5 | 28.4 | 24.2 |
| 12 | | | 29.7 | 24.7 |
| 14 | | | 30.2 | 25.2 |
| 16 | | | 30.4 | 25.7 |
| 18 | | | 30.6 | 26.4 |
| 20 | | | 31.0 | 26.8 |
| 22 | | | 31.9 | 27.4 |
| 24 | | | 31.6 | 27.8 |
| 26 | | | 32.5 | |

Streptomycin levels in urine were determined by the same procedure as for blood. When high levels were anticipated (above 25 units per c.c.), the urine was diluted with water.

Data for standard curves for four patients treated with streptomycin are presented to illustrate the assay method and to demonstrate the necessity for individual standard curves (see Table I). Fig. 3 shows a typical standard curve. The semilogarithm standard curves on the blood and urine of Patient E-2 (Fig. 4 and 4*A*) were used to estimate the very high drug levels. Although some work was completed dealing with the dilution of bloods containing high unitages of streptomycin (40 units per cubic centimeter and above), sufficient data were not accumulated to warrant recommendations. Results plotted on semilogarithm paper show the curve to be a straight line function, and it is reasonable to assume that, for all practical purposes, such high unitages can be determined.

## APPLICATION OF ASSAY TECHNIQUE

Application of this method for determining streptomycin levels in man and in various laboratory animals is discussed in a succeeding report.[15] This method has also been applied successfully to the assay of streptothricin and other antibiotics. This method for streptomycin assay was adopted by the Office of the Surgeon General, United States Army, on September, 1945, for use in Army hospitals.

## SUMMARY

A method for the quantitative determination of streptomycin and other antibiotics in body fluids has been described in detail.

The authors wish to express their gratitude to Prof. H. E. Carter and associates, for permission to use the original formula for streptothricin assay agar; to Merck & Company, Inc., New York, N. Y., for the supply of streptomycin; to Anna E. Funk, for suggesting the use of an automatic syringe filler; and to Lieutenant Colonel Murray Sanders, for his kind suggestions and careful guidance.

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# STUDIES ON STREPTOMYCIN*

## II. Blood Levels and Urinary Excretion in Man and Animals

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With the Technical Assistance of L. A. Collins, PhM3c, C. F. Redmon, PhM2c, and M. C. Chambers, PhM2c

IN INVESTIGATIONS of the chemotherapeutic activity of streptomycin, both in laboratory animals and man, basic information was needed concerning absorption of the drug by various routes and its distribution and excretion in several species of animals. At the inauguration of the experiments herein reported, a limited amount of information, chiefly toxicologic, was provided by Merck Institute for Therapeutic Research.[1] More recently, data of other investigators have been reported in the literature.[2, 3] A limited amount of additional information on the behavior of the drug in man is available but has not yet reached general circulation.[4]

A study of streptomycin in a variety of animal species was carried out preliminary to, or during, experiments designed to test therapeutic effectiveness of the antibiotic in specific bacterial infections. In addition, the use of the drug for treatment of infection in hospitalized patients provided an opportunity for comparison of results of animal experiments with the findings in man. However, for present purposes, data relative to the effect of the antibiotic on the infectious diseases have been excluded from this report. In all cases, whole blood, with an oxalate anticoagulant, was used for streptomycin determinations performed according to the method previously described.[5]

### PROCEDURE AND RESULTS

*Parenteral Administration.*—Streptomycin blood levels were determined in the mouse, guinea pig, rabbit, monkey, and man. The drug was administered by subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal injection.

*Mice:* Animals were weighed to the nearest 0.5 gram, and graded doses of streptomycin in sterile distilled water were administered with a tuberculin syringe. The concentration of streptomycin solution was adjusted so that doses of approximately constant volume were given. Following administration, blood samples were obtained by heart puncture from the etherized or chloroform-anesthetized animal, and at the same time extracts of the liver, spleen, and kidneys were made by trituration of each organ with sterile water and sand followed

These studies were conducted at Camp Detrick, Md., from December, 1944, to October, 1945. Received for publication, Feb. 20, 1946.

*Streptomycin as referred to in this paper is streptomycin hydrochloride and was supplied by Merck & Company, Inc., New York, N. Y.

TABLE I. STREPTOMYCIN LEVELS IN BLOOD AND TISSUE EXTRACTS OF MICE* FOLLOWING INTRAMUSCULAR INJECTION OF THE DRUG

| TIME (HOURS) | DOSE, 5,000 UNITS PER KG. | | | | DOSE, 10,000 UNITS PER KG. | | | |
|---|---|---|---|---|---|---|---|---|
| | BLOOD LEVEL (UNITS PER C.C.) | | KIDNEY (UNITS PER ORGAN) | | BLOOD LEVEL (UNITS PER C.C.) | | KIDNEY (UNITS PER ORGAN) | |
| | EXP. 1 | EXP. 2 | EXP. 1 | EXP. 2 | EXP. 1 | EXP. 2 | EXP. 1 | EXP. 2 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ⅛ | >10 | 8 | 5.0 | <2 | 8 | 8.5 | <0.5 | <2 |
| | >10 | 9 | <0.5 | <2 | 8 | 6.5 | <0.5 | <2 |
| ⅛ | >10 | 7.5 | 0.5 | <2 | 8 | 8.5 | <0.5 | <2 |
| | >10 | 6.5 | <0.5 | <2 | 8 | 6.5 | <0.5 | <2 |
| ½ | >10 | 6.5 | <0.5 | <2 | 6.5 | 8.0 | <0.5 | <2 |
| | >10 | 6.5 | <0.5 | 0 | 6.5 | 6.5 | <0.5 | <2 |
| ¾ | 6.5 | 1.5 | 0 | <2 | 2.0 | 6.5 | <0.5 | <2 |
| | 4.0 | 1.5 | <0.5 | 0 | 2.5 | | <0.5 | <2 |
| 1 | 5.0 | 5.0 | <0.5 | <2 | 4.0 | 3.5 | <0.5 | <2 |
| | 3.0 | 0.5 | 0 | <2 | 6.0 | 3.0 | <0.5 | <2 |
| 1¼ | 3.0 | 2.0 | 0 | 0 | 2.0 | 1.0 | 0 | <2 |
| | 3.0 | 0.5 | 0 | 0 | <0.5 | 3.0 | 0 | <2 |
| 1½ | <2.5 | 0.5 | 0 | 0 | 2.5 | 2.0 | 0 | 0 |
| | 2 | <0.5 | 0 | 0 | <0.5 | 0.5 | <0.5 | <2 |
| 1¾ | 2.5 | <0.5 | 0 | 0 | <0.5 | <0.5 | 0 | <2 |
| | <2 | <0.5 | 0 | 0 | 0 | 1.5 | 0 | <2 |
| 2 | <2 | <0.5 | <0.5 | 0 | <0.5 | 0 | 0 | 0 |
| | <2 | 0 | 0 | 0 | 0 | 0 | 0 | <2 |
| 2¼ | <2 | 0 | 0 | 0 | <0.5 | 0 | 0 | <2 |
| | <2 | 0 | 0 | 0 | <0.5 | 0 | <0.5 | <2 |
| 2½ | 0 | | | 0 | | 0 | | <2 |
| | 0 | | | 0 | | 0 | | <2 |
| 2¾ | 0 | 0 | 0 | 0 | <0.5 | | 0 | <2 |
| | 0 | 0 | 0 | 0 | <0.5 | | 0 | <2 |
| 3 | 0 | 0 | 0 | 0 | <0.5 | 0 | 0 | <2 |
| | 0 | 0 | 0 | 0 | <0.5 | 0 | 0 | <2 |
| 3½ | 0 | | | <2 | | 0 | | <2 |
| | 0 | | | <2 | | 0 | | <2 |
| 4 | 0 | | | 0 | | 0 | | <2 |
| | 0 | | | 0 | | 0 | | <2 |
| 4½ | 0 | | | 0 | | 0 | | <2 |
| | 0 | | | 0 | | 0 | | <2 |
| 5 | 0 | | | <2 | | 0 | | <2 |
| | 0 | | | 0 | | 0 | | <2 |
| 5½ | 0 | | | 0 | | 0 | | <2 |
| | 0 | | | 0 | | 0 | | <2 |
| 6 | 0 | | | 0 | | 0 | | <2 |
| | 0 | | | 0 | | 0 | | <2 |
| | 0 | | | <2 | | 0 | | <2 |

*Extracts of liver and spleens negative in every case.

by centrifugation (1 c.c. water used for spleen and kidney; 2 c.c., for liver). Two animals were sacrificed for each time interval, and separate determinations were made. The tissue extracts were assayed against standard curves of normal tissue extract supernatants of like concentration.

The streptomycin levels in the blood and tissue extracts of mice, following intramuscular administration of the drug, are given in Table I. From Table I it can be seen that doses of 5,000 and 10,000 units per kilogram of streptomycin in mice produced a maximum blood level of 8 units per cubic centimeter, or above, and that the blood concentration of the drug dropped rapidly and disappeared about two hours after the injection. Of the organs assayed, the liver and spleen were negative at all times, and the kidney concentration was so low as to indicate that the drug was merely in a state preparatory to elimination.

TABLE II. BLOOD LEVELS OF STREPTOMYCIN IN GUINEA PIGS AFTER SINGLE DOSE ADMINISTRATION

| METHOD OF ADMINISTRATION | I.D. | I.P. | S.C. | S.C. | I.D. | I.P. | I.M. | S.C. | S.C. | S.C. | S.C. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DOSE (UNITS PER KILOGRAM) | 2500 | 2500 | 3000 | 5000 | 5000 | 5000 | 10,000 | 15,000 | 17,500 | 20,000 | 22,500 |
| TIME (HOURS) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) | BLOOD LEVEL (UNITS PER C.C.) |
| 0 | 0 | 0 | | 5 | <5 | 0 | | | | | |
| | 2.0 | 1.0 | | | 0.5 | | 5.5 | | | | |
| ¼ | | | | | | | 5.5 | | | | |
| | | | | | | | 8.5 | | | | |
| | 3.0 | 2.0 | 6.5 | 1.2 | 3.0 | 3.0 | 6.5 | 17.0 | 20.0 | 20.0 | 18.5 |
| ½ | | | 5.5 | | | | 6.0 | 15.0 | | 17.0 | 19.5 |
| | | | 4.5 | | | | 10.0 | 12.0 | | 14.0 | |
| | 4.0 | | | | 3.0 | 3.5 | 6.7 | | | | |
| ¾ | | | | | | | 6.0 | | | | |
| | | | | | | | 8.5 | | | | |
| | | 3.0 | 5.2 | 4.7 | 4.5 | 4.0 | 5.5 | 21.0 | 26.0 | 20.5 | 22.5 |
| 1 | | | 5.7 | | | | 7.0 | 15.0 | | 21.5 | 26.5 |
| | | | 6.5 | | | | 9.0 | | | 15.0 | |
| | 4.0 | | | | | | | | 20.5 | 23.0 | 26.0 |
| 1¼ | | | | | | | 7.5 | 19.0 | | 23.0 | 23.0 |
| | | | | | | | >10.0 | | | 20.5 | |
| | 2.5 | 2.5 | 4.7 | 4.7 | 6.5 | 4.5 | 6.5 | 21.7 | 25.0 | 21.5 | 26.5 |
| 1½ | | | 4.7 | | | | 6.5 | 15.0 | | 23.5 | 26.5 |
| | | | 7.2 | | | | >10.0 | | | 17.0 | |
| | 3.5 | | 4.5 | 3.7 | | 3.5 | 8.0 | 16.3 | 20.5 | 23.0 | 23.0 |
| 2 | | | 4.5 | | | | 7.0 | 11.5 | | 21.5 | |
| | | | 7.2 | | | | | 16.0 | | 19.0 | |
| | | | | | 5.5 | | 8.0 | | | | |
| 2¼ | | | | | | | 8.0 | 18.5 | | 19.0 | |
| | | | | | | | | 16.0 | | 23.0 | |
| | | | | 5.5 | | 1.0 | 7.5 | | | | |
| 2½ | | | | | | | 7.0 | 16.0 | | 19.5 | |
| | | | | | | | | | | 20.5 | |
| | 3.0 | | 4.5 | 4.0 | 5.0 | | 5.5 | | | | |
| 3 | | | 3.2 | | | | | | | | |
| | | | 4.7 | | | | | 13.5 | | | |
| | 3.0 | 1.5 | 2.5 | 2.5 | | | 7.5 | | | | |
| 3½ | | | 3.2 | | | | | | | | |
| | | | 7.0 | | | | | | | | |
| 4 | 3.0 | 1.0 | 3.5 | 2.7 | | 2.5 | 7.5 | | | | |
| | | | 2.2 | | | | | | | | |
| | 3.0 | 1.0 | 2.5 | 3.2 | | 1.5 | 6.5 | | | | |
| 4½ | | | 2.2 | | | | | | | | |
| | | | 3.2 | | | | | | | | |
| | | | 2.7 | 4.0 | | 6.5 | | | | | |
| 5 | | | 3.7 | | | | | | | | |
| | | | 5.5 | | | | | | | | |
| | | | 4.5 | 2.5 | | | 5.5 | | | | |
| 5½ | | | 4.7 | | | | | | | | |
| | | | 2.7 | | | | | | | | |
| 6 | 1.5 | | 4.0 | | | | 6.0 | | | | |
| | | | 5.0 | | | | | | | | |

I.D., Intradermal; I.P., Intraperitoneal; S.C., Subcutaneous; I.M., Intramuscular.

Lungs, spleen, and liver extracts prepared and found negative. Kidney extracts positive to extent of approximately from 5 to 15 units per organ.

*Guinea Pigs:* Animals were weighed to the nearest 5 grams, and streptomycin in sterile distilled water or sterile 0.85 per cent saline solution was administered by tuberculin syringe. Blood samples for assay were obtained at varying intervals by heart puncture, generally under pentobarbital sodium anesthesia, which was found not to affect the test. Check runs were made without anesthesia. In order to maintain blood volume, 5 per cent glucose in 0.85 per cent saline solution was given to the animals at periodic intervals. When using large animals, individual standard curves were prepared for each, but this proved undesirable for smaller guinea pigs due to blood depletion. For such animals, a standard curve of pooled guinea pig blood was prepared and used for several animals. Blood levels of streptomycin in guinea pigs, following administration of graded doses of the drug by various routes, are given in Table II. These results indicate that the rate of absorption of streptomycin varied with the route of administration. Variations in blood levels were observed with changes in dosage, but the relationship was not directly proportional. The drug was found in the blood as late as six hours after injection.

TABLE III. TYPICAL BLOOD LEVELS IN RABBITS FOLLOWING INTRAMUSCULAR INJECTION OF 10,000 UNITS PER KILOGRAM OF STREPTOMYCIN

| RABBIT 3A81 | | | |
|---|---|---|---|
| HOURS AFTER INJECTION | STREPTOMYCIN BLOOD LEVEL* (UNITS PER C.C.) | HOURS AFTER INJECTION | STREPTOMYCIN BLOOD LEVEL (UNITS PER C.C.) |
| 0.5 | >10 | 3.5 | 4.5 |
| 1 | >10 | 4 | 4.0 |
| 1.5 | >10 | 4.5 | 3.5 |
| 2 | 6.2 | 5 | 4.0 |
| 2.5 | 5 | 5.5 | 2.5 |
| 3 | 4 | 6 | 1.5 |

*Blood level determined in oxalated whole blood.

*Rabbits:* Although the rabbit has been the chief source of supply for blood when checking standard curves for various changes in the assay method, comparatively few data have been obtained on correlation of blood level with dosages of streptomycin. However, the available data indicate that the rabbit reacts to streptomycin more like the guinea pig than the mouse. Thus, it appears to be able to retain the drug sufficiently to permit administration at from three- to four-hour intervals. Typical streptomycin blood levels obtained in a rabbit following intramuscular injection of 10,000 units per kilogram are given in Table III and were found to range from above 10 units per cubic centimeter at one-half hour to 1.5 units per cubic centimeter at six hours. Blood samples for analysis were obtained from the marginal ear vein.

*Monkeys:* Two animals were injected intramuscularly with 1,250 units per kilogram of streptomycin. The blood of one of these animals was assayed for streptomycin levels at frequent intervals for three hours, and the second animal was studied for six hours. Results are given in Table IV and indicate that the monkey blood level ranged from 2.5 units per cubic centimeter at fifteen minutes to less than 0.5 unit per cubic centimeter at five hours after the injection, with later samples being negative.

TABLE IV. BLOOD LEVELS IN TWO MONKEYS FOLLOWING INTRAMUSCULAR INJECTION OF 1,250 UNITS PER KILOGRAM OF STREPTOMYCIN

| HOURS AFTER INJECTION | STREPTOMYCIN BLOOD LEVELS* | |
|---|---|---|
| | MONKEY 49 | MONKEY 50 |
| 0.25 | † | 2.7 |
| 0.5 | | 2.5 |
| 0.75 | | 2.2 |
| 1 | 1.25 | 1.7 |
| 1.5 | 0.75 | 1.5 |
| 2 | 0.5 | 1.0 |
| 2.5 | 0.4 | 1.0 |
| 3 | 0.4 | 0.7 |
| 3.5 | 0.4 | |
| 4 | 0.4 | |
| 4.5 | 0.4 | |
| 5 | 0.4 | |
| 6 | 0 | |

*Blood level determinations made on oxalated whole blood.
†Determination not made.

TABLE V. AVERAGE (SIX PADS) ZONE INHIBITION OF STREPTOMYCIN IN BLOOD OF EXPERIMENTALLY INFECTED MONKEYS; FIGURES FOR PLOTTING INDIVIDUAL MONKEY STANDARD CURVES (MM.)

| STREPTOMYCIN ADDED (UNITS PER C.C.) | DIAMETER ZONE INHIBITION (MM.) MONKEY | | | | | | |
|---|---|---|---|---|---|---|---|
| | 49 | 57 | 61 | 63 | 66 | 67 | 71 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.2 | T* | 16.5 | 16.7 | 16.8 | 16.6 | T | T |
| 0.5 | 17.4 | 17.9 | 19.2 | | 18.7 | 17.7 | 17.4 |
| 1.0 | 20.3 | 20.1 | 20.5 | 21.3 | 19.8 | 19.7 | 19.6 |
| 2.0 | 21.9 | † | 21.3 | | 21.5 | 21.4 | 21.7 |
| 3.0 | 22.7 | 23.4 | 22.2 | 23.9 | 22.0 | 22.7 | 23.0 |
| 4.0 | 23.4 | | 22.5 | | 22.8 | | 23.5 |
| 5.0 | 23.7 | 24.1 | | 24.9 | 23.5 | 24.1 | 24.0 |
| 6.0 | 24.3 | | 23.7 | | 23.8 | | 24.5 |
| 8.0 | 24.8 | 24.6 | | 25.4 | 24.0 | | |

*T. Trace quantity.
†Number of points determined for standard curve limited by quantity of blood available.

Following these experiments, streptomycin blood levels were followed in seven monkeys, five of which had been experimentally infected, the remainder serving as drug controls. The same dose as mentioned, that is, 1,250 units per kilogram, was given every three hours for nine days by intramuscular injection. In Table V are presented the data used for establishing the individual standard curves for each monkey and the individual variability in reaction between blood and streptomycin is shown. In Table VI are shown the daily blood levels of the animals and in Table VII, the rate of disappearance of the drug from the blood stream of individual animals after the last injection. Additional evidence of the individual reaction of animals to streptomycin with variation in daily blood levels, as well as in the rate of disappearance of the drug, is also shown in Tables VI and VII.

*Man:* In the preliminary studies several standard curves were made for normal human beings, but the blood level of streptomycin from parenteral administration was followed in only four patients, and urinary excretion was determined on two of these patients. In Table VIII is summarized the strepto-

TABLE VI. BLOOD LEVELS OF EXPERIMENTALLY INFECTED AND CONTROL MONKEYS RECEIVING STREPTOMYCIN THERAPY* (TREATMENT, STREPTOMYCIN 1,250 UNITS PER KILOGRAM INTRAMUSCULARLY EVERY THREE HOURS FOR NINE DAYS; DETERMINATIONS MADE FOLLOWING 8 A.M. INJECTION EXCEPT AS NOTED)

| DAY OF THERAPY | MONKEY 49† | | | MONKEY 61† | | | MONKEY 71 | | | MONKEY 63 | | | MONKEY 57 | | | MONKEY 66 | | | MONKEY 67 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TIME AFTER INJECTION | | UNITS | TIME AFTER INJECTION | | UNITS | TIME AFTER INJECTION | | UNITS | TIME AFTER INJECTION | | UNITS | TIME AFTER INJECTION | | UNITS | TIME AFTER INJECTION | | UNITS | TIME AFTER INJECTION | | UNITS |
| | HR. | MIN. | PER C.C. | HR. | MIN. | PER C.C. | HR. | MIN. | PER C.C. | HR. | MIN. | PER C.C. | HR. | MIN. | PER C.C. | HR. | MIN. | PER C.C. | HR. | MIN. | PER C.C. |
| 5/13/45 | | 7 | 3.8 | | 19 | >6(7.0)‡ | | 35 | 2.6 | | 43 | 1.6 | | 54 | 4.0 | 1 | 1 | 3.1 | 1 | 10 | 2 |
| 1 | 2 | 16 | 2.3 | 2 | 24 | 2.9 | 2 | 30 | 1.9 | 2 | 37 | 0.9 | 2 | 45 | 1.2 | 2 | 53 | 1.5 | 3 | 10§ | 1 |
| 5/14/45 | | | | | | | | | | | | | | | | | | | | | |
| 2 | | 10 | 3.9 | | 20 | >6(7.7) | | 26 | 4.2 | | 42 | 4.1 | | 50 | 4.4 | 1 | 6 | 3.7 | 1 | 15 | 3.7 |
| 5/15/45 | | | | | | | | | | | | | | | | | | | | | |
| 3 | | 20 | 8.0 | | 27 | >6(10) | | 33 | 6.0 | | 40 | 4.1 | | 50 | 8.0 | 1 | 5 | 6.0 | 1 | 10 | 4.6 |
| 5/16/45 | 2 | 20 | 3.3 | 2 | 37 | 3.6 | 2 | 24 | 2.4 | 2 | 28 | 1.2 | 2 | 32 | 2.1 | 2 | 45 | 1.7 | 2 | 55 | 3.2 |
| 4 | | 10 | 8.0 | | 35 | >6(10) | | 17 | 5.5 | | 25 | 5.3 | | 30 | 3.6 | | 40 | 5.7 | | 55 | 4.6 |
| 5/17/45 | 1 | 9 | 4.2 | | 34 | 5.5 | | 14 | 4.0 | | 20 | 2.5 | | 30 | 2.7 | | 40 | 6.8 | | 45 | 4.1 |
| 5 | 2 | 35 | 1.2 | 2 | 52 | 2.2 | 2 | 40 | 1.2 | 2 | 45 | 0.9 | 2 | 49 | 1.2 | 2 | 58 | 1.5 | 3 | | 1.7 |
| 5/18/45 | | | | | | | | | | | | | | | | | | | | | |
| 6 | | 5 | 3.2 | | 11 | >6(9.0) | | 28 | 3.0 | | 40 | 2.3 | | 17 | 3.5 | | 55 | 5.3 | 1 | 5 | 2.7 |
| 5/19/45 | | 13 | 4.5 | | 20 | >6(9.0) | | 25 | 6(7.5) | | 31 | 2.3 | | 40 | 3.4 | | 48 | 5.0 | 1 | | 4.3 |
| 7 | 2 | 32‖ | 0.9 | 2 | 37‖ | 0.7 | 2 | 27‖ | 2.0 | 2 | 47‖ | 0.7 | 2 | 43‖ | 1.7 | 2 | 55‖ | 1.1 | 3‖ | | 2.2 |
| 5/20/45 | | | | | | | | | | | | | | | | | | | | | |
| 8 | | 10 | 7.1 | | 15 | >6(8.0) | | 22 | 3.5 | | 30 | 2.6 | | 37 | 3.6 | | 47 | 4.1 | | 55 | 3.7 |
| 5/21/45 | | 10 | 4.1 | | 15 | >6(8.0) | | 18 | 5.2 | | 25 | 5.6 | | 30 | 5.8 | | 35 | 8(8.3) | | 42 | 4.2 |
| 9 | 2 | 45 | 1.5 | 2 | 50 | 1.6 | 2 | 54 | 1.5 | 2 | 58 | 0.5 | 3 | 2§ | 1.0 | 2 | 53 | 0.7 | 3 | 15§ | 1.6 |

*Blood of all animals negative for *Bacillus subtilis* inhibition immediately prior to treatment.
†Uninfected animals.
‡Blood levels above highest point of standard curve; the figure in parenthesis represents best estimate.
§Time more than three hours due to from five to fifteen-minute period necessary for injection.
‖Units per c.c. after 11:00 A.M. injection; remainder from 8:00 A.M. injection.

TABLE VII. RATE OF DISAPPEARANCE OF STREPTOMYCIN FROM BLOOD OF MONKEYS AFTER LAST INTRAMUSCULAR INJECTION OF DRUG; BLOOD LEVELS IN UNITS PER CUBIC CENTIMETER

| MONKEY | TIME AFTER LAST INJECTION (HR.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 3.0 | 6.0 | 9.0 | 12 | 18 | 24 |
| 57 | 5.8 | 1.0 | 0.6 | T* | 0 | 0 | 0 |
| 61 | >6 (8.0) | 1.6 | 0.6 | <0.5 | T | T | T |
| 63 | 5.6 | 0.5 | <0.5 | T | T | 0 | 0 |
| 66 | >8 (8.3) | 0.7 | T | T | 0 | 0 | 0 |
| 67 | 4.2 | 1.6 | 0.7 | 0 | 0 | 0 | 0 |

*T, Trace quantity.

TABLE VIII. STREPTOMYCIN BLOOD LEVELS IN PATIENTS UNDER INTENSIVE THERAPY COURSE OF DRUG*

| PATIENT ONE | | | PATIENT TWO | | | PATIENT THREE | | | PATIENT FOUR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DAY OF TREATMENT | HOURS AFTER INJECTION | BLOOD LEVEL (UNITS PER C.C.) | DAY OF TREATMENT | HOURS AFTER INJECTION | BLOOD LEVEL (UNITS PER C.C.) | DAY OF TREATMENT | HOURS AFTER INJECTION | BLOOD LEVEL (UNITS PER C.C.) | DAY OF TREATMENT | HOURS AFTER INJECTION | BLOOD LEVEL (UNITS PER C.C.) |
| 1 | ½† | 1.2 | | | | 1 | 1† | 6.0 | 1 | 1† | 1.1 |
| | 1† | 2.0 | | | | | 2† | 6.0 | | 2† | 1.9 |
| | 2† | 1.0 | | | | | 3† | 4.0 | | 3† | 2.6 |
| | 3† | 1.1 | | | | | 1 | 8.5 | | 1 | 2.0 |
| | ½ | 2.1 | | | | | | | | | |
| 2 | 3 | 1.2 | 2 | 3 | 1.3 | 2 | 3 | 8.0 | 2 | 3 | 2.3 |
| | 1 | 1.6 | | ¼ | 2.1 | | 1 | 9.0 | | ¾ | 7.4 |
| | | | | ½ | 3.1 | | | | | | |
| | | | | ¾ | 2.1 | | | | | | |
| | | | | 1 | 2.5 | | | | | | |
| | | | | 1½ | 1.6 | | | | | | |
| | | | | 3 | 1.0 | | | | | | |
| 3 | 1 | 2.1 | 3 | 2½ | 1.1 | 3 | 3 | 10.5 | 3 | 3 | 3.6 |
| | 3 | 1.6 | | ¾ | 1.0 | | 1⅓ | 11.0 | | ¾ | 7.4 |
| | ¾ | 2.0 | | | | | | | | | |
| | | | | | | | | | 4 | 3 | 2.0 |
| | | | | | | | | | | 1⅙ | 3.6 |
| 5 | 3 | 1.0 | | | | | | | 5 | 2 | 8.5 |
| | 1 | 1.4 | | | | | | | | 3 | 4.0 |
| | | | | | | | | | 6 | 3 | 3.1 |
| | | | | | | | | | | 1 | 7.2 |
| | | | | | | | | | | 3 | 4.8 |
| | | | | | | | | | | 1 | 5.3 |
| 7 | 3 | 0.9 | | | | | | | 8 | 3 | 5.7 |
| | 5 | 0.7 | | | | | | | | 5 | 2.8 |
| | 7 | 0.6 | | | | | | | | 7 | 1.9 |
| | 9 | <0.5 | | | | | | | | 9 | 1.7 |
| | | | | | | | | | | 28 | Trace |

*Courses of therapy were as follows:

Patient 1, treatment started 10 A.M., May 8, 1945; 30,000 units intramuscularly every three hours. Stopped May 14, 1945. Total dose, 1,440,000 units. Lot 181.

Patient 2, treatment started noon, April 18, 1945; 30,000 units intramuscularly every three hours. Stopped 6 P.M., April 21, 1945. Total dose, 810,000 units. Lot 4R307L.

Patient 3, treatment started 11 A.M., May 19, 1945; 250,000 units intramuscularly every three hours. May 23, 1945; 250,000 units intravenously noon and 3 P.M.; 500,000 units intravenously, drip; May 24, 1945, 500,000 units intravenously, drip, midnight. Then 1,000,000 units intravenously, drip every six hours to 8 P.M., May 26, 1945, at which time last dose of 500,000 units intravenously was given. Total dose, 15,750,000 units. Lot 212.

Patient 4, treatment started 10 A.M., May 21, 1945; 100,000 units intramuscularly every three hours. Stopped 7 A.M., May 28, 1945. Total dose, 5,600,000 units. Lot 212.

†Determinations following first dose of drug.

TABLE IX. DAILY URINARY EXCRETION OF STREPTOMYCIN BY TWO PATIENTS DURING AND AFTER INTENSIVE THERAPY*

| DAY AFTER TREATMENT BEGUN | PATIENT 3 | | PATIENT 4 | |
|---|---|---|---|---|
| | URINE VOL. 24 HR. (C.C.) | URINARY EXCRETION (UNITS) | URINE VOL. 24 HR. (C.C.) | URINARY EXCRETION (UNITS) |
| 1 | 675 | 241,383 | 1,830 | 819,000 |
| 2 | 510 | 218,700 | 925† | 509,083 |
| 3 | 940 | 677,980 | 1,300 | 552,933 |
| 4 | 1,700 | 744,600 | 1,550 | 888,666 |
| 5 | 2,950 | 1,923,400 | 1,825 | 773,219 |
| 6 | | ‡ | 1,400 | 645,766 |
| 7 | 1,350 | 578,700 | 1,730 | 625,107 |
| 8 | 1,300§ | 180,266 | 1,700§ | 727,600 |
| 9 | 1,100 | 23,137 | 1,350 | 179,550 |
| 10 | 2,900 | 21,605 | 1,200 | 27,040 |
| 11 | 1,900 | 18,113 | 1,350 | 11,250 |
| 12 | 1,450 | 5,520 | 1,400 | 5,600 |
| 13 | 2,150 | 7,525 | 1,375 | 9,625 |
| 14 | 2,500 | 9,750 | 2,025 | 8,100 |
| 15 | 1,700 | 5,780 | 1,500 | 5,550 |
| 16 | 1,750 | 4,200 | 1,050 | 1,890 |
| 17 | 1,550 | 4,340 | 1,650 | 2,805 |
| 18 | 1,750 | 3,150 | 2,000 | 1,800 |
| 19 | | ‡ | 2,100 | 2,205 |
| 20 | 1,850 | 3,700 | 1,500 | 1,350 |
| 21 | 2,020 | 4,243 | 1,800 | 2,340 |
| 22 | 1,350 | 1,350 | 1,575 | T‖ |
| 23 | 1,400 | 2,520 | 1,375 | T‖ |
| 24 | 2,000 | 1,200 | 1,425 | T‖ |
| 25 | 2,000 | 1,600 | 850 | T‖ |
| 26 | 2,000 | ‡ | | |
| 27 | 2,300 | 7,130 | | |
| 28 | 2,390 | 1,195 | | |
| 29 | | 0 | | |
| Total recovered | | 4,691,087 | | 5,800,479 |
| Total adm. | | 15,570,000 | | 5,600,000 |

*For course of therapy, see footnote* of Table VIII.
†Twelve hour sample.
‡No determination made.
§Treatment stopped.
‖Trace; impossible to read accurately due to urinary contamination of Pseudomonos sp. that was also inhibitory to *Bacillus subtilis*.

mycin blood levels of four patients under treatment, the clinical results of which will be reported elsewhere. Daily urinary excretions of two patients are given in Table IX. The results presented in Table VIII indicate that in human beings a dose of 30,000 units of streptomycin every three hours by intramuscular injection would provide a blood level of from 1 to 2.5 units per cubic centimeter and that the blood level can be increased by increasing the dose. In Table IX it is shown that large amounts of the drug are eliminated by the kidney, but with daily variations. Further, urinary contamination interferes with the accuracy of the method.

In order to check the assay methods statistically for correlation from human being to human being, the data used for establishing thirteen individual standard curves are presented in Table X, and a statistical analysis of these data for points up through 10 units per cubic centimeter is shown in Table XI. Although doses now used for therapy frequently result in blood levels higher than 10 units per cubic centimeter in human beings, the data presented indicate that the

TABLE X. DATA FOR PREPARATION OF STANDARD CURVE FOR THIRTEEN HUMAN BEINGS

| PATIENT | QUANTITY STREPTOMYCIN ADDED TO WHOLE BLOOD (UNITS PER CUBIC CENTIMETER) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | 8.0 | 10.0 |
| 1 | 17.4 | 18.8 | 20.0 | 22.1 | 22.8 | 23.0 | 23.7 | 24.6 | 25.0 |
| 2 | 16.3 | 18.4 | 19.9 | 20.9 | 21.5 | 22.2 | 23.0 | 23.4 | 23.3 |
| 3 | 17.4 | 18.9 | 20.3 | 21.5 | 22.4 | 23.2 | 24.1 | 23.9 | 24.5 |
| 4 | 18.7 | 19.9 | 21.4 | 22.1 | * | 22.7 | | 23.8 | |
| 5 | 18.2 | 19.3 | 21.2 | 22.1 | 22.8 | 23.5 | 24.1 | | |
| 6 | 16.6 | 17.9 | 20.2 | 21.2 | 22.0 | 23.3 | 23.1 | 23.5 | 24.6 |
| 7 | 15.8 | 18.2 | 20.4 | 21.5 | 21.8 | 22.0 | | 22.4 | 22.6 |
| 8 | Trace | 19.3 | | 22.7 | | 24.4 | 26.1 | 25.4 | 27.2 |
| 9 | 16.0 | 20.0 | 21.1 | 21.7 | 22.4 | 22.6 | 23.4 | 24.9 | 25.5 |
| 10 | 18.0 | 21.1 | 23.4 | 24.5 | 25.5 | 25.1 | 25.4 | 25.3 | 26.4 |
| 11 | 15.1 | 20.8 | 24.0 | 24.3 | 26.9 | 27.9 | 27.6 | 28.7 | |
| 12 | 16.4 | 18.2 | 22.5 | 24.3 | | 25.5 | 26.2 | 27.4 | 28.4 |
| 13 | 0 | 15.8 | 18.1 | 20.5 | 21.6 | 22.3 | 22.4 | 23.0 | 24.2 |

*Determination not made.

TABLE XI. STATISTICAL ANALYSIS OF DATA USED IN ESTABLISHING STANDARD CURVES FOR THIRTEEN HUMAN BEINGS*

| CONCENTRATION | NUMBER OF OBSERVATIONS | STANDARD DEVIATION | STANDARD ERROR OF MEANS | MEANS | MEANS MINUS 2 STANDARD ERRORS | MEANS PLUS 2 STANDARD ERRORS | COEFFICIENT OF VARIATION |
|---|---|---|---|---|---|---|---|
| 0.5 | 11 | 1.154 | 0.348 | 16.945 | 16.249 | 17.641 | 6.81 |
| 1.0 | 13 | 1.377 | 0.382 | 18.969 | 18.205 | 19.733 | 7.26 |
| 2.0 | 12 | 1.632 | 0.471 | 21.042 | 20.100 | 21.984 | 7.76 |
| 3.0 | 13 | 1.328 | 0.368 | 22.262 | 21.526 | 22.998 | 5.96 |
| 4.0 | 10 | 1.792 | 0.567 | 22.970 | 21.836 | 24.104 | 7.80 |
| 5.0 | 13 | 1.672 | 0.464 | 23.669 | 22.741 | 24.597 | 7.06 |
| 6.0 | 11 | 1.632 | 0.492 | 24.464 | 23.480 | 25.448 | 6.67 |
| 8.0 | 12 | 1.833 | 0.529 | 24.692 | 23.634 | 25.750 | 7.42 |
| 10.0 | 10 | 1.764 | 0.558 | 25.170 | 24.054 | 26.286 | 7.01 |

*Courtesy of Ensign M. R. Zelle.

method is reliable, and to date standard curves through 24 units per cubic centimeter have been made without serious difficulty.

*Oral Administration.*—To test the absorption of streptomycin from the gastrointestinal tract, the drug was given orally to mice, rabbits, dogs, and man.

*Mice:* For oral administration to mice, a tuberculin syringe fitted with a long, smoothly blunt, 18-gauge needle was used. The drug was dissolved in sterile distilled water to a concentration such that a mouse weighing 20 grams would receive 0.5 c.c. Animals receiving the same quantity of saline by the oral route were sacrificed for controls. Blood samples were obtained by heart puncture of anesthetized animals 0.25, 0.5, 1.0, and 1.5 hours after administration. In no case was streptomycin detected in the blood of the twenty animals given the drug orally.

*Rabbits:* Two animals were given 10,000 units per kilogram of streptomycin mixed with oil of theobroma, U. S. P., and contained in gelatine capsules. For administration, a stomach tube of sufficient diameter to pass the capsule was used. Blood samples were obtained from the marginal ear vein at frequent intervals. At no time was a detectable quantity of streptomycin found in the blood of either rabbit.

TABLE XII. ORAL ADMINISTRATION OF STREPTOMYCIN IN DOGS USING VARIOUS VEHICLES; NO SIGNIFICANT BLOOD LEVELS ATTAINED

| DOG | SEX | WEIGHT (KG.) | DOSE (UNITS PER KG.) | OBSERVATION TIME (HR.) | VEHICLE FOR ADMINISTRATION OF DRUG |
|---|---|---|---|---|---|
| B | M | 12 | 2,000 | 6 | Oil of theobroma plus alcohol |
| L | F | 20 | 6,250 | 6.5 | Oil of theobroma plus alcohol |
| B | M | 12 | 6,000 | 7 | Oil of theobroma |
| L | F | 20 | 6,000 | 7 | Oil of theobroma |
| L | F | 20 | 10,000 | 3 | Amphojel,* 5 c.c., 15 min. before drug (drug as powder in gelatin capsule) |
| P | M | 21 | 10,000 | 3.5 | Amphojel,* 5 c.c., 15 min. before drug in oil of theobroma plus alcohol |
| L | F | 20 | 10,000 | 5 | Acacia emulsion (O/W) of corn oil, $H_2O$, alcohol |
| P | M | 21 | 10,000 | 5 | Drug dissolved in milk |
| J | M | 8 | 10,000 | 5 | $H_2O$ containing 1 Gm. disodium phosphate |
| R | F | 10 | 10,000 | 5 | $H_2O$ containing 1 Gm. sodium citrate |
| M | M | 8 | 10,000 | 6 | $H_2O$ plus alcohol (1 c.c.) |
| G | F | 8 | 10,000 | 6 | $H_2O$ plus alcohol (2 c.c.) |
| B | M | 12 | 10,000 | 6 | $H_2O$ plus alcohol (3 c.c.) |
| J | M | 8 | 10,000 | 7 | Lanolin emulsion (W/O) of corn oil, water |
| G | F | 8 | 10,000 | 7 | Lanolin emulsion (W/O) of corn oil, water |
| R | F | 10 | 10,000 | 6 | Yolk of egg |
| L | F | 20 | 10,000 | 6 | White of egg |
| J | M | 10 | 10,000 | 6 | White of egg |
| G | F | 10 | 10,000 | 6 | Yolk of egg |

*John Wyeth & Brother, Inc., Philadelphia, Pa.

*Dogs:* Seven dogs were employed to test the suitability of several vehicles for aiding absorption of streptomycin after oral administration, and the results are summarized in Table XII. In no case was a detectable quantity of streptomycin found in the blood, but random urine samples from untrained dogs showed detectable and often considerable urine levels of the drug.

*Man:* One volunteer was administered 500,000 units (6,666 units per kilogram) of streptomycin orally in each of two doses three hours apart. The drug was mixed with oil of theobroma, U. S. P., and inclosed in gelatin capsules. Blood samples 0.5, 1, 2, and 3 hours after each dose were negative for streptomycin, but hourly urine samples did demonstrate a factor inhibitory to *Bacillus subtilis* that was not found before administration of the drug.

## DISCUSSION

The investigations indicate that there is not only a species variation in reaction to streptomycin, but also a considerable variation in individuals of a given species. Thus, it is considered that the mouse is a poor choice of animal for work requiring maintenance of streptomycin blood levels. This is also believed to be true of this animal in respect to other antibiotic work. How the drug is eliminated or destroyed by the mouse at a faster rate than by other animals has not been determined, but examination of the extracts of the liver and spleen indicate that it is not removed by, or stored in, either of these organs. Its appearance in the kidney extracts is attributed to the filtration and concentration procedures of urinary elimination.

For correlation with man, the guinea pig appears to be the more desirable choice of small animal for antibiotic tests. However, it appears probable that

the guinea pig does tend to permit cumulation of the drug to an extent greater than that which has been seen in man. Thus, a dose of 15,000 units per kilogram every three hours was recommended for work requiring maintenance of a blood level of 20 units per cubic centimeter or above in the guinea pig. On trial, the repeated doses were found to provide such a level or above, although single dose administration did not always provide the minimum concentration. Experience to date indicates that in all animals a minimum of three consecutive doses at the proposed therapy interval should be used for obtaining preliminary data on the expected blood level of a drug course.

The rabbit, judging from the very scanty data available, appears to be a satisfactory small animal for studying streptomycin blood levels. It is probable that the blood level is depleted more slowly in the rabbit than in most other animals, and if prolonged intervals are desired between consecutive doses of the drug, the guinea pig or rabbit appears to be the animal of choice.

The experiment with monkeys provided the assay method with its first significant test for day-to-day determination. The infected animals were definitely ill at the beginning of therapy, and, although blood levels did vary from animal to animal and from day to day in the same animal, the data are considered essentially correct. Some of the standard curves were checked at the conclusion of the experiment after the blood had been found free of the drug, and these corresponded very well with the originals. The rate of disappearance of the drug from the blood of each animal was considered additional evidence that the animal variation is considerable. Thus, it is believed that the best streptomycin therapy will result from variation in dose, to maintain a rather narrow blood level range, instead of from the administration of a predetermined dose of the drug.

In man the blood levels seemed to be within the range expected from previous animal work. In the one patient (Patient 4) receiving the dose of drug nearest that administered to the monkey, the blood levels compared closely. The striking findings from human administration were the great concentration of the drug in its passage through the kidney and the prolonged urinary excretion after cessation of therapy. No explanation is advanced for this observation, but certainly it is logical to conclude that a storage of drug is established somewhere in the body. The experience gained so far in attempting to dilute blood and protein solutions containing the drug indicates that streptomycin has a definite affinity for protein, but whether the drug is stored in a protein-rich portion of the body or whether it is only slowly removed from the blood proteins has not been established. Either of these conditions might provide an explanation for prolonged urinary excretion.

Due to the discomfort of continued intramuscular, subcutaneous, or intravenous injection of the drug, and with the hope of eliminating some of the toxicity from the first lots of the drug, oral administration was attempted. Experiments based on penicillin studies were tried, and insignificant absorption of streptomycin from the gastrointestinal tract was demonstrated. To date no satisfactory vehicle for oral administration is available. Due to scarcity of the drug, the work was stopped until more of its chemical and physical character-

istics which can be used as a base for different approaches are known. The consistent finding of the drug in the urine of the dog and man indicates that the problem of oral administration is not insurmountable, and certainly the therapeutic advantage gained would justify further investigation.

Red and white blood counts were followed in some of the rabbits, guinea pigs, monkeys, and human beings during their exposure to streptomycin, and in no case was an abnormal result obtained. Preliminary studies on the partition coefficient of streptomycin in blood indicate that the cells do not carry the drug, since neither water nor saline washing of the centrifuged cells or clot provided an antibiotic material, although both plasma and serum were active. This partition was true whether the streptomycin was added to drawn blood or blood samples were taken from an animal following drug administration.

## SUMMARY

1. Streptomycin was administered by subcutaneous, intravenous, intradermal, intramuscular, and intraperitoneal injection to the mouse, guinea pig, rabbit, and man, and blood levels of the drug were determined at various intervals after administration. Maximum blood levels appeared from fifteen to sixty minutes following its injection.

2. For maintenance of blood levels of the drug following one dose, the animal of choice appeared to be, in order, guinea pig, rabbit, monkey, and mouse.

3. Urinary excretion of streptomycin was followed in two patients on an intensive parenteral therapy course of the drug and in one volunteer receiving the drug orally.

4. Administration of streptomycin apparently does not affect the count of red or white blood cells.

5. Therapeutic blood levels of the drug could not be obtained by oral administration in a variety of vehicles.

6. Variation of blood levels from a given dose of streptomycin is seen from animal to animal, and during a therapeutic course, the blood level in the same animal varies from day to day. Similar variation is seen in disappearance of the drug from the blood at the end of the drug course. This pattern is also followed in human beings.

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# A STUDY OF THE DIFFUSION OF PENICILLIN ACROSS THE SEROUS MEMBRANES OF JOINT CAVITIES

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MANY infections of joints are caused by bacteria which are sensitive to penicillin. As a prerequisite to the establishment of a plan of therapy for the purulent arthritides, we felt that it was necessary first of all to determine whether there was an exchange of penicillin between the blood and joint fluid. The results of this study are included in the present paper.

## PLAN OF STUDY

Penicillin was injected into a knee joint or administered systemically to a group of patients, and assays were performed on joint fluid and serum of each patient to determine the amount of penicillin diffusing across the serous membrane of the joint cavity. Similar studies were done while the patient was under treatment in a fever cabinet to ascertain whether high temperatures would influence the exchange of penicillin between joint fluid and blood. Specimens for penicillin assay on these patients were taken after the patient had been in the cabinet for several hours and the body temperature was 104° F. or over.

The patients studied had a variety of joint diseases: rheumatic fever or rheumatoid, gonococcic, and other pyogenic arthritides. In order to determine whether there was any difference in the diffusion of penicillin into joint fluid from the blood in diseased and normal joints, several patients receiving penicillin, but with no evidence of joint disease, where given up to 100 c.c. of physiologic salt solution into a knee joint, and this fluid was used for penicillin assay.

Several preparations of penicillin were employed: the sodium and calcium† salts of penicillin G, the calcium salts† of penicillin X, and sodium crystalline penicillin.‡ Penicillin was given orally in tablets† in doses of 100,000 units every two hours; by continuous intravenous infusion in doses of from 100,000 to 200,000 units for periods of from six to twelve hours; and by intermittent intramuscular injection, using from 20,000 to 80,000 units every two hours, from 25,000 to 30,000 units every three hours, and 50,000 units every six hours. When penicillin was injected into a knee joint, up to 200,000 units were dissolved in from 20 to 50 c.c. of physiologic salt solution. Joint fluid for penicillin assay was obtained by aspiration through a spinal needle which had previously been inserted into the knee joint and taped in place for the necessary number of hours. At the desired time the stylet was removed and joint fluid withdrawn. Penicillin

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*Dr. Feffer's contribution to this study was made prior to his entrance into the Armed Forces.

†Supplied by Lederle Laboratories, Inc., Pearl River, N. Y.

‡Supplied by Merck & Company, Rahway, N. J.

TABLE I. SERUM AND KNEE FLUID CONCENTRATIONS OF PENICIL

| PATIENT | JOINT DISEASE | DOSE (UNITS) | | CONCENTRATIONS OF PENICIL | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 5 MIN. | 15 MIN. | 30 MIN. | 1 HR. | 2 HR. | 3 HR. | 4 HR. | 5 HR. |
| B. B. | Pyogenic arthritis | 30,000 | Serum | | | | .078 | .078 | .078 | .078 | .078 |
| | | | Knee fluid | 40 | | | | | | | |
| C. M. | Pyogenic arthritis | 30,000 | Serum | | | | | .039 | | .039 | |
| | | | Knee fluid | 20 | | | | | | | |
| T. K. | Gonococcic arthritis | 50,000 | Serum | | | | .039 | .039 | .039 | .039 | .039 |
| | | | Knee fluid | 40 | | | | | | | |
| C. J. | Gonococcic arthritis | 50,000 | Serum | | | | .039 | .039 | .039 | .039 | .039 |
| | | | Knee fluid | 40 | | | | | | | |
| S. A. | Gonococcic arthritis | 50,000 | Serum | | | | | .078 | | .156 | |
| | | | Knee fluid | | | | | 10 | | 5 | |
| L. J. | Gonococcic arthritis | 50,000 | Serum | | | | | .039 | | .156 | |
| | | | Knee fluid | | | | | 10 | | 5 | |
| L. J.* | Gonococcic arthritis | 50,000 | Serum | | | | | .039 | | .039 | |
| | | | Knee fluid | | | | | 40 | | 40 | |
| Lu. L. | Pyogenic arthritis | 50,000 | Serum | | | | .078 | .312 | .312 | | |
| | | | Knee fluid | | | | 40 | 40 | 40 | | |
| J. B. | Pyogenic arthritis | 50,000 | Serum | | | | .078 | | .156 | | |
| | | | Knee fluid | | | | 20 | | 20 | | |
| R. C. | Rheumatic fever | 50,000 | Serum | 0 | 0 | .0195 | .039 | .156 | .312 | .156 | .078 |
| | | | Knee fluid | 40 | | | | | | | |
| C. M. | Pyogenic arthritis | 60,000 | Serum | 0 | | | | .0195 | | .0195 | |
| | | | Knee fluid | 40 | | | | | | | |
| T. K. | Gonococcic arthritis | 100,000 | Serum | | | | .078 | .039 | .039 | .039 | .039 |
| | | | Knee fluid | 40 | | | | | | | |
| A. K. | Rheumatoid arthritis | 100,000 | Serum | | | | .0195 | .0195 | .0195 | .0195 | .0195 |
| | | | Knee fluid | 40 | | | | | | | |
| T. K. | Gonococcic arthritis | 200,000 | Serum | | | | .039 | .039 | .039 | .039 | .039 |
| | | | Knee fluid | | | | | | | | |
| A. K. | Arthralgia | 200,000 | Serum | | | | 0 | | 0 | | |
| | | | Knee fluid | | | | | | 20 | | |
| La. L. | Arthralgia | 200,000 | Serum | | | | 0 | | 0 | | |
| | | | Knee fluid | | | | | | 20 | | |

*Fever therapy.

determinations were done on serum and joint fluid at regular intervals by the methods of Rammelkamp[1] and Randall and associates.[2] No differentiation is made as to the type of penicillin employed because the results were the same with all preparations.

## RESULTS

Of the fourteen patients who were given doses of from 30,000 to 200,000 units of penicillin into a knee joint on sixteen occasions, penicillin was found to be present in the knee fluid for from twenty-four to one hundred twenty hours, depending upon the dose (Fig. 1). It was present in the blood on fourteen occasions one hour after intra-articular injection. In one patient, from whom blood was taken at one-half hour after injection into the knee joint, penicillin was present. In each patient the penicillin concentration in the blood was maintained at a nearly constant level throughout the period of observation. The heights of the serum concentrations are shown in Table I. Two patients had a median serum penicillin concentration of .0195 unit per cubic centimeter, five had .039 unit per cubic centimeter, and six had .078 unit per cubic centimeter or better. Although knee fluid concentrations of penicillin of from 10 to 40 units per cubic centimeter were maintained for periods of twelve and twenty-four hours,

PENICILLIN FOLLOWING INTRA-ARTICULAR INJECTION OF PENICILLIN

| PENICILLIN (UNITS PER CUBIC CENTIMETER) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 HR. | 6 HR. | 7 HR. | 8 HR. | 9 HR. | 10 HR. | 11 HR. | 12 HR. | 18 HR. | 24 HR. | 48 HR. | 72 HR. | 96 HR. | 108 HR. | 120 HR. | 144 HR. |
| .078 | .078 | .078 | .078 | .078 | .039 | .039 | .039 | 0 | 0 | 0 | 0 | | | | |
| | | | | | | | .625 | | | | | | | | |
| | | | .039 | | | | .039 | .039 | 0 | 0 | | | | | |
| | | | | | | | .625 | | .078 | 0 | | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | | .039 | 0 | 0 | 0 | | | | | |
| | | | | | | | .156 | | 0 | 0 | | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | 0 | 0 | 0 | | | | | |
| | 20 | | | | | | 2.5 | | .312 | 0 | | | | | |
| | .078 | | | | | | | | | | | | | | |
| | 2.5 | | | | | | | | | | | | | | |
| | .312 | | .156 | | .078 | | .039 | | | | | | | | |
| | 5 | | | | | | | | | | | | | | |
| | .039 | | | | | | | | | | | | | | |
| | 20 | | | | | | | | | | | | | | |
| | .156 | | | | | | | | | | | | | | |
| | 10 | | | | | | | | | | | | | | |
| .078 | .078 | .078 | | | | | 0 | | | | | | | | |
| | 10 | | | | | | 5 | | | | | | | | |
| | | | .0195 | | | | .0195 | .0195 | .0195 | .0195 | 0 | | | | |
| | | | | | | | | | 5 | .312 | .0195 | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | 0 | 0 | | | |
| | | | | | | | 40 | | 10 | 1.25 | .156 | .039 | | | |
| .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | 0 | | | |
| | | | | | | | 20 | | 20 | 2.5 | 2.5 | 0 | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | | .0195 | 0 |
| | | | | | | | 20 | | 20 | 2.5 | | 1.25 | | .156 | 0 |
| | 0 | | | 0 | | | 0 | | 0 | 0 | | | | | |
| | 10 | | | | | | 5 | | 2.5 | .312 | .156 | .156 | 0 | | |
| | 0 | | | 0 | | | 0 | | 0 | 0 | | | | | |
| | 10 | | | | | | 5 | | 2.5 | .625 | .312 | .078 | 0 | | |

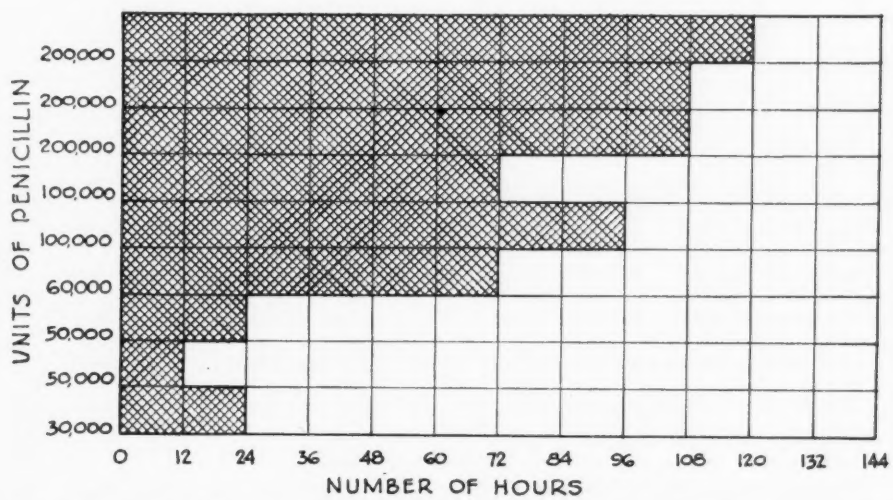


Fig. 1.—Duration of detectable penicillin concentration in knee joint fluid following intra-articular injection.

TABLE II. KNEE FLUID AND SERUM PENICILLIN CONCENTRATIONS FOLLOW

| PATIENT | JOINT DISEASE | DOSE (UNITS) | NUMBER OF DOSES | METHOD OF ADMINISTRATION | | 15 MIN. | 30 MIN | 1 HR. |
|---|---|---|---|---|---|---|---|---|
| C. J. | Gonococcic arthritis | 20,000 q. 2 hr. | 4 | Intramuscular | Knee fluid | | | .0193 |
| | | | | | Serum | | | .156 |
| B. T. | Gonococcic arthritis | 20,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | | | .0193 |
| | | | | | Serum | | | .078 |
| B. T.* | Gonococcic arthritis | 20,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .312 |
| B. T. | Gonococcic arthritis | 20,000 q. 2 hr. | 1 | Intramuscular | Knee fluid | | | .0193 |
| | | | | | Serum | | | .078 |
| G. C. | Gonococcic arthritis | 20,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .078 |
| G. C.* | Gonococcic arthritis | 20,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .156 |
| H. B. | Gonococcic arthritis | 20,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | | .625 | .156 |
| | | | | | Serum | | 2.5 | .312 |
| B. J. | Gonococcic arthritis | 20,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | 0 | | .312 |
| | | | | | Serum | .625 | | .078 |
| R. C. | Rheumatic fever | 50,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | .0195 | .039 | .078 |
| | | | | | Serum | 1.25 | .625 | .312 |
| M. J. | Rheumatic fever | 50,000 q. 2 hr. | 2 | Intramuscular | Knee fluid | .0195 | .078 | .078 |
| | | | | | Serum | .625 | | .312 |
| C. M. | Gonococcic arthritis | 60,000 q. 2 hr. | 4 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .625 |
| L. P. | Gonococcic arthritis | 80,000 q. 2 hr. | 6 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | 2.5 |
| E. B. | Normal | 25,000 q. 3 hr. | 2 | Intramuscular | Knee fluid | | | 0 |
| | | | | | Serum | | | .078 |
| E. B.* | Normal | 25,000 q. 3 hr. | 2 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .156 |
| E. B. | Normal | 25,000 q. 3 hr. | 2 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .078 |
| R. M. | Gonococcic arthritis | 25,000 q. 3 hr. | 2 | Intramuscular | Knee fluid | | .078 | .156 |
| | | | | | Serum | | 1.25 | .625 |
| L. C. | Gonococcic arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .078 |
| | | | | | Serum | | | .625 |
| O. W. | Gonococcic arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .078 |
| | | | | | Serum | | | .156 |
| O. W. | Gonococcic arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .078 |
| | | | | | Serum | | | .156 |
| O. W. | Gonococcic arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .078 |
| | | | | | Serum | | | .312 |
| C. A. | Gonococcic arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .312 |
| O. J. | Gonococcic arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .078 |
| | | | | | Serum | | | .156 |
| M. J. | Rheumatoid arthritis | 25,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | .156 |
| L. F. | Gonococcic arthritis | 30,000 q. 3 hr. | 1 | Intramuscular | Knee fluid | | | .078 |
| | | | | | Serum | | | .625 |
| T. K. | Gonococcic arthritis | 40,000 q. 4 hr. | 3 | Intramuscular | Knee fluid | | | .0195 |
| | | | | | Serum | | | .039 |
| S. A. | Gonococcic arthritis | 50,000 q. 6 hr. | 1 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | |
| S. A.* | Gonococcic arthritis | 50,000 q. 6 hr. | 1 | Intramuscular | Knee fluid | | | .312 |
| | | | | | Serum | | | |
| S. A. | Gonococcic arthritis | 50,000 q. 6 hr. | 1 | Intramuscular | Knee fluid | | | .039 |
| | | | | | Serum | | | |
| L. J. | Gonococcic arthritis | 50,000 q. 6 hr. | 1 | Intramuscular | Knee fluid | | | |
| | | | | | Serum | | | |
| L. J.* | Gonococcic arthritis | 50,000 q. 6 hr. | 1 | Intramuscular | Knee fluid | | | |
| | | | | | Serum | | | |
| L. J. | Gonococcic arthritis | 50,000 q. 6 hr. | 1 | Intramuscular | Knee fluid | | | |
| | | | | | Serum | | | |
| B. S. | Pyogenic arthritis | 100,000 continuous 6 hr. | 1 | Intravenous | Knee fluid | | | .039 |
| | | | | | Serum | | | .156 |

*Fever therapy.

FOLLOWING PARENTERAL AND ORAL ADMINISTRATION OF PENICILLIN

| CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 HR. | 2 HR. | 3 HR. | 4 HR. | 5 HR. | 6 HR. | 7 HR. | 8 HR. | 9 HR. | 10 HR. | 11 HR. | 12 HR. | 13 HR. |
| .0195 | .039 | .039 | .039 | .039 | .039 | .039 | .0195 | | | | | |
| .156 | .0195 | .156 | .039 | .312 | .039 | .0195 | 0 | | | | | |
| .0195 | .0195 | .0195 | .0195 | | | | | | | | | |
| .078 | .039 | .156 | .039 | | | | | | | | | |
| .039 | .078 | .078 | .078 | | | | | | | | | |
| .312 | .039 | .078 | .039 | | | | | | | | | |
| .0195 | .0195 | | | | | | | | | | | |
| .078 | .039 | | | | | | | | | | | |
| .039 | .039 | .039 | .039 | | | | | | | | | |
| .078 | 0 | .078 | .078 | | | | | | | | | |
| .039 | .039 | 0 | .039 | | | | | | | | | |
| .156 | .039 | .078 | 0 | | | | | | | | | |
| .156 | .039 | .039 | .039 | | | | | | | | | |
| .312 | .039 | .625 | .039 | | | | | | | | | |
| .312 | .078 | .078 | .078 | | | | | | | | | |
| .078 | 0 | .156 | .039 | | | | | | | | | |
| .078 | .078 | .156 | .156 | .156 | .156 | .078 | .039 | .0195 | 0 | | | |
| .312 | .156 | | .156 | | .039 | | 0 | | | | | |
| .078 | .078 | .156 | .078 | .078 | .078 | .0195 | 0 | | | | | |
| .312 | .156 | .625 | .078 | | .039 | | 0 | | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .0195 | .0195 | | | |
| .625 | .156 | 1.25 | .156 | 1.25 | .156 | .312 | .039 | 0 | | | | |
| .039 | .039 | .039 | .039 | .078 | .039 | .039 | .039 | .078 | .078 | .039 | .039 | |
| 2.5 | .156 | 1.25 | .078 | 2.5 | .156 | .625 | .078 | .625 | .078 | 1.25 | .156 | |
| 0 | .039 | .039 | .078 | .039 | .039 | .039 | .039 | | | | | |
| .078 | .039 | .039 | .156 | .078 | .039 | 0 | 0 | | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | | | | | |
| .156 | .078 | .039 | .312 | .078 | .078 | 0 | 0 | | | | | |
| .039 | .039 | .039 | .039 | .039 | .078 | .039 | 0 | | | | | |
| .078 | .039 | 0 | .156 | .039 | 0 | 0 | 0 | | | | | |
| .156 | .078 | .039 | .078 | | | | | | | | | |
| .625 | .078 | .039 | 2.5 | | | | | | | | | |
| .078 | .039 | 0 | | | | | | | | | | |
| .625 | .156 | .078 | | | | | | | | | | |
| .078 | .078 | .078 | | | | | | | | | | |
| .156 | .078 | .039 | | | | | | | | | | |
| .078 | .078 | .156 | | | | | | | | | | |
| .156 | .156 | .039 | | | | | | | | | | |
| .078 | .156 | .156 | | | | | | | | | | |
| .312 | .156 | .039 | | | | | | | | | | |
| .039 | .078 | .078 | | | | | | | | | | |
| .312 | .156 | .039 | | | | | | | | | | |
| .078 | .078 | .078 | | | | | | | | | | |
| .156 | .078 | 0 | | | | | | | | | | |
| .039 | .078 | .078 | | | | | | | | | | |
| .156 | .039 | 0 | | | | | | | | | | |
| .078 | .078 | .156 | | | | | | | | | | |
| .625 | .039 | 0 | | | | | | | | | | |
| .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 |
| .039 | 0 | 0 | 0 | .078 | .0195 | .0195 | 0 | .078 | .0195 | 0 | 0 | |
| .039 | .039 | .039 | .039 | .039 | .039 | | | | | | | |
| | 1.25 | | .312 | | .078 | | | | | | | |
| .312 | .625 | .625 | .625 | .625 | .625 | .625 | | | | | | |
| | .625 | | .312 | | .156 | | | | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | | | | | | | |
| | .625 | | .312 | | .078 | | | | | | | |
| | .039 | | .039 | | .039 | | | | | | | |
| | .156 | | .078 | | .039 | | | | | | | |
| | .039 | | .039 | | .039 | | | | | | | |
| | .312 | | .078 | | .039 | | | | | | | |
| | .039 | | .039 | | .039 | | | | | | | |
| | .156 | | .078 | | .039 | | | | | | | |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | | | | | |
| .156 | .312 | .312 | .312 | .312 | .312 | .156 | .0195 | | | | | |

TABLE II—

| PATIENT | JOINT DISEASE | DOSE (UNITS) | NUMBER OF DOSES | METHOD OF ADMINISTRATION | | 15 MIN. | 30 MIN |
|---|---|---|---|---|---|---|---|
| B. S. | Pyogenic arthritis | 200,000 continuous 6 hr. | I | Intravenous | Knee fluid<br>Serum | | |
| T. K. | Gonococcic arthritis | 200,000 continuous 12 hr. | I | Intravenous | Knee fluid<br>Serum | | |
| A. K. | Rheumatoid arthritis | 200,000 continuous 12 hr. | I | Intravenous | Knee fluid<br>Serum | | |
| T. K. | Gonococcic arthritis | 200,000 continuous 8 hr. | I | Intravenous | Knee fluid<br>Serum | | |
| J. H. | Gonococcic arthritis | 100,000 q. 2 hr. | 2 | Oral | Knee fluid<br>Serum | | |

the highest serum concentration observed was .312 unit per cubic centimeter. In no instance was there evidence of absorption of penicillin from the joint fluid into the blood when the concentration in the knee fluid fell below .156 unit per cubic centimeter. Two patients with arthralgias secondary to a systemic disease showed no evidence of absorption of penicillin from the knee joint into the blood following an intra-articular injection of 200,000 units.

Of a group of twenty-two patients who were given penicillin orally or parenterally on thirty-seven occasions, all had significant concentrations in their sera. Penicillin was found to have diffused into the joint fluid in all patients except the one who was given the drug orally (Table II). Several patients had no joint disease, and the injected physiologic salt solution was used as joint fluid. As in the case of intra-articular injection, the absorption of parenterally administered penicillin into the joint was almost constant, with median concentrations of penicillin of .0195 unit per cubic centimeter of joint fluid in six instances, .039 unit per cubic centimeter in fifteen, and .078 unit per cubic centimeter or better in fifteen. The amount of penicillin which diffused into the joint fluid was usually independent of the serum concentration. Joint fluid concentrations of penicillin varied up to 50 per cent of the highest serum concentration. Occasionally the joint fluid concentration was higher than the serum concentration when the patient was receiving intermittent intramuscular injections, demonstrating that the drug apparently was excreted more slowly from the joint fluid than from the blood. We feel that these results represented an adequate sampling, as blood and joint fluid for penicillin assay were taken at one- or two-hour intervals for from six to twelve hours in twenty studies, for four hours in seven, and for two or three hours in ten.

In about one-half of the studies the blood and joint fluid were taken directly following the start of penicillin therapy, and in the other one-half the studies were performed after the patient had been under treatment for one or more days. The results were the same regardless of the duration of therapy indicating that penicillin did not accumulate in joint fluid.

Penicillin was found to diffuse rapidly into the joint fluid when given systemically. Of the three patients from whom joint fluid was obtained fifteen minutes after the first parenteral injection, penicillin was present in the joint fluid of two. In the other patients, penicillin was found in the first specimen, which was obtained one-half and one hour after the drug was started.

CONT'D

| CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 HR. | 2 HR. | 3 HR. | 4 HR. | 5 HR. | 6 HR. | 7 HR. | 8 HR. | 9 HR. | 10 HR. | 11 HR. | 12 HR. | 13 HR. |
| .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | | | | | |
| .312 | .625 | .625 | .625 | .625 | .625 | .039 | .0195 | | | | | |
| .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .039 | .039 | .0195 | .039 | .039 | .039 | .039 |
| .078 | .039 | .0195 | .0195 | .0195 | .0195 | .312 | .078 | .0195 | .078 | .039 | .039 | 0 |
| .0195 | .0195 | .0195 | .0195 | .0195 | .0195 | .039 | .039 | .0195 | .039 | .039 | .039 | .0195 |
| .156 | .156 | .156 | .156 | .156 | .156 | .156 | .156 | .312 | .312 | .312 | .312 | .0195 |
| .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | .039 | | | |
| 1.25 | 2.5 | 2.5 | 5 | 5 | 1.25 | 1.25 | .312 | .0195 | 0 | | | |
| 0 | 0 | 0 | 0 | | | | | | | | | |
| .078 | 0 | .078 | 0 | | | | | | | | | |

In several patients determinations were made on blood and serum for several hours after the drug was discontinued, and the joint fluid was found to contain penicillin for from one to three hours longer than the blood, corroborating the observation that penicillin was excreted more slowly from the joint fluid than from the blood.

The effects of fever therapy in the exchange of penicillin between joint fluid and blood were studied in six patients. A comparison was made in each patient of the amount of penicillin transferring between the blood and joint fluid before, during, and after fever therapy. One patient received the penicillin intra-articularly and five were given the drug intramuscularly. In two of the latter group absorption into the knee fluid was found to have occurred in slightly higher concentrations during fever therapy than under normal conditions. However, fever therapy did not influence the amount of penicillin transferred across the serous membranes in the remaining four patients.

Penicillin has been found to be irritating to serous membranes, causing a pleocytosis on injection into the spinal fluid[3] or pleural space.[4] We found that such a reaction occurred when penicillin was injected into a joint. There was an increase in the number of white blood cells over a forty-eight hour period which receded to normal over the next few days.

## DISCUSSION

Rammelkamp and Keefer[5] found serum penicillin concentrations up to .007 unit per cubic centimeter following an injection of 10,000 units of the antibiotic into a knee joint or bursa. Florey and Heatley[6] demonstrated absorption of penicillin into the blood for from seven to twenty-four hours following an intra-articular injection of from 100,000 to 120,000 units of the drug. However, they did not report any serum concentrations. Herrell and associates[7] noted that up to 50 per cent of the serum concentrations of penicillin will diffuse into joint fluid with parenteral administration. Ory and co-workers[8] were able to detect penicillin concentrations of .03 and .06 unit per cubic centimeter of joint fluid from one-half to two and one-half hours following intramuscular injection of from 15,000 to 20,000 units. Balboni and associates[9] reported consistently significant levels of penicillin in the joint fluid of seven patients following the intramuscular injection of from 25,000 to 40,000 units of penicillin.

The joint fluid levels reported by these investigators are higher than those obtained by Ory and co-workers and by us with comparative doses.

Although we were able to demonstrate the transfer of penicillin between blood and joint fluid, analysis of our results shows that when penicillin was given systemically, joint fluid concentrations of .0195 unit per cubic centimeter were found in 16 per cent of the studies, .039 unit per cubic centimeter in 41 per cent, and .078 unit per cubic centimeter or better in 41 per cent. The highest joint concentration of penicillin obtained was .625 unit per cubic centimeter in two patients on one each of a series of determinations. When the penicillin was injected into a knee joint, absorption into the blood reached a level of .0195 unit per cubic centimeter in 15 per cent of the patients, .039 unit per cubic centimeter in 39 per cent, and .078 unit per cubic centimeter in 46 per cent. The greatest amount of penicillin absorbed into the blood from the joint space was .312 unit per cubic centimeter.

When the joint fluid concentrations of penicillin obtained with systemic administration of the drug are correlated with the concentration usually needed to kill the various penicillin-sensitive bacteria, particularly staphylococci, it is found that in a significant percentage of instances insufficient amounts of penicillin diffuse into the joint fluid to control the infection of the joint. Similarly, insufficient amounts of penicillin are absorbed into the blood from a joint space to care for other sites of infection. These observations are confirmed by our clinical experiences with patients who had pyogenic arthritis and periarticular inflammation and/or bacteriemia in whom penicillin had to be given systemically as well as intra-articularly to control the extra-articular infection. We have also found systemic penicillin alone to be inadequate in the treatment of pyogenic arthritis complicating a bacteriemia.

Correlation of various factors such as the height of serum concentrations of penicillin, duration of treatment, method of administration, and type of joint disease, with the amount of penicillin diffusing across the serous membranes of joints, reveals that although there is a tendency toward greater diffusion with high serum or joint fluid concentrations, there is no constant relationship between the two. Neither the duration of treatment nor the method of administration influenced the transfer of penicillin across the serous membrane. Except in two patients with arthralgia, who had no absorption of penicillin from the joint into the blood, we found the diffusion across the serous membrane to be the same irrespective of the presence or type of joint disease. Fever therapy did not significantly alter the diffusion of penicillin, for in only two of six patients was there a slight increase in the amount of penicillin exchanged between joint fluid and blood.

## SUMMARY AND CONCLUSIONS

1. Penicillin will diffuse across the serous membranes of joints when given systemically or intra-articularly.

2. Low concentration of penicillin in the joint fluid following the systemic administration and in the blood following intra-articular injection were obtained in a significant number of studies.

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# OUTBREAK OF HEMOLYTIC STREPTOCOCCUS THROAT INFECTION CONTROLLED BY SULFADIAZINE

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THE prophylactic use of sulfonamides against bacterial infections has assumed considerable importance during recent years. Sulfanilamide has been used in preventing streptococcal upper respiratory infections and relapses in rheumatic fever.[1-3] Kuhns and associates[4] found that sulfadiazine in amounts as low as 2 Gm. daily diminished the meningococcal carrier rate as well as the incidence of meningococcal meningitis. Rubenstein and Foley[5] studied two outbreaks of scarlet fever, one occurring in an orphanage due to type 1 streptococcus and the other occurring in a schoolroom due to a type 5 streptococcus. In both epidemics small daily doses of sulfonamides reduced to a minimum the number of persons carrying the incriminated organism.

During the 1944-1945 holiday season an epidemic of sore throats occurred among the staff of a United States Army General Hospital on Oahu, Territory of Hawaii. Due to the burden of casualties from the Philippine Campaign at this time, it was necessary to take quick and effective action in order to maintain operation of the hospital. This was carried out successfully with therapeutic and prophylactic use of sulfadiazine.

The first patient was admitted Dec. 28, 1944. Two were admitted Dec. 31, 1944, and three more, Jan. 1, 1945. On Jan. 2, 1945, eight more of the hospital personnel were admitted at various times of the day. The earlier individuals showed the presence of large numbers of beta hemolytic streptococci in throat cultures. It was apparent that a fairly large epidemic of streptococcic sore throat was going to disable the staff of the hospital, unless the proper action were taken immediately. The protection of the hospital patients was also of prime importance. In order to control the situation quickly the following measures were taken: (1) detection of all cases and suspected cases by screening of the personnel; (2) isolation of all cases and suspects; (3) efforts to determine the origin of all known cases; and (4) administration of sulfadiazine prophylactically to personnel of the staff.

These measures were accomplished in the following manner. A team of medical officers examined all enlisted personnel, by roster, as they passed through the mess line. All men were questioned as to how they felt; their noses and throats were examined; and their temperatures were determined when indicated. All officers and nurses were examined after lunch at the outpatient clinic. Through this process twenty-one new patients, of whom two were nurses, were admitted to the hospital and placed in contagious wards.

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The epidemiology of the outbreak was investigated. To aid in this study, a map of the hospital area was prepared, and all cases were marked on the map as to the location of the patient's work and his sleeping quarters. Throat cultures were performed on all mess personnel to detect the presence of carriers. Histories on each case were carefully checked for a common source. The evidence gathered by these methods of approach all pointed to a probable origin at a dance held by the enlisted men of the organization Dec. 27, 1944. Of the WAC personnel who attended the dance, two were admitted with the disease.

Prophylactic administration of sulfadiazine was started Jan. 3, 1945, and continued for five days by giving the enlisted personnel a tablet (7½ gr. of sulfadiazine) before each meal under the supervision of the noncommissioned officers. Tablets were made available to the officers and nurses at the mess hall, and they were instructed to take one tablet three times daily. Following the administration of the drug, a marked decrease in the incidence of sore throats was noted. Between Dec. 28. 1844, and Jan. 3, 1945, there was a total of thirty-five patient admitted with hemolytic streptococcus sore throats. Following the administration of sulfadiazine prophylaxis, which started Jan. 3, 1945, there were only seven cases up to Jan. 15, 1945, when the last case occurred. No reactions due to sensitivity to the sulfadiazine were noted.

## TREATMENT

Patients with the more severe cases were given an initial dose of 4 Gm. of sulfadiazine followed by 1 Gm. every four hours. This was maintained for periods varying from two to eight days. Response to therapy was excellent. Some of the patients admitted were not very ill and were treated symptomatically. Those with mild cases responded well, even without the use of sulfonamides. Of the entire group there was only one patient who was seriously ill. He had a profuse pharyngitis with considerable edema of the fauces and bilateral cervical adenopathy. On sulfadiazine, his temperature dropped from 103° F. to normal in seventy-two hours, and he made an uneventful recovery.

## CLINICAL LABORATORY FINDINGS

Throat cultures were made daily from individuals suspected of hemolytic streptococcus infection. Culture media in each case were a blood agar plate and Löffler's serum medium. The throats at time of culture in the positive cases were uniformly inflamed. Pure cultures of hemolytic streptococci were usually found in these individuals. In one moderately severe case a mixture of *Staphylococcus aureus* and *Streptococcus pyogenes* was found initially. After a few days' treatment with sulfadiazine the hemolytic streptococci began to disappear, and indifferent and green-producing streptococci, micrococci, and Neisseria appeared in the cultures. During convalescence, positive cultures would frequently recur after several negative daily cultures. Two patients had clinical relapses.

The leucocyte count on admission varied over a broad range from 5,000 to 20,000. In general, the leucocyte count did not correlate very closely with the clinical severity. Urinary findings were essentially normal.

## CULTURAL AND SEROLOGIC STUDIES

Twenty-six strains of hemolytic streptococci isolated from patients in this outbreak were studied in detail. Lancefield precipitin methods were employed for serologic analysis.

*Methods and Materials.*—Cultures were isolated on horse blood agar plates. Hemolysis, if its type were questionable, was checked in deep agar plates. One strain showed a minute zone of beta hemolysis and proved to belong to Group H (Table I).

TABLE I. DISTRIBUTION OF STREPTOCOCCUS GROUPS AND TYPES IN TWENTY-SIX STRAINS OF HEMOLYTIC STREPTOCOCCI STUDIED

| GROUP* | NUMBER | PER CENT | TYPE† | NUMBER | PER CENT |
|---|---|---|---|---|---|
| A | 25 | 96 | 3 | 1 | 4 |
| H | 1 | 4 | 36 | 18 | 72 |
| | | | 38 | 2 | 8 |
| | | | NT‡ | 4 | 16 |
| Total | | | | 25 | 100 |

*Groups of sera employed: A, B, C, D, E, F, G, H, K, L.
†Types of sera employed: 1, 3, 5, 6, 10-12, 14, 17, 18, 19, 24, 26, 28, 30, 31, 36, 37, 38, 42, 43.
‡No type obtained in any serum.

Extracts for grouping and typing were prepared from a single broth culture. Forty cubic centimeters of tryptose phosphate broth at pH 7.6 to 7.8 were heavily inoculated with a young culture of streptococcus. Care was taken that incubator temperature did not rise over 37° C. during growing of the broth culture, since higher temperatures might prevent formation of M-substance.[6] At eighteen hours' incubation, the culture was centrifuged, and the packed cells were resuspended in 0.6 c.c. of N/20 HCl. The suspension was mixed, heated in boiling water for ten minutes with shaking, cooled quickly, and one drop of phenol red indicator solution added. One drop of N/5 NaOH was added to reduce final volume of extract; then neutralization was completed to a pink color with N/20 NaOH made up in M/60 phosphate buffer at pH 7.0 (0.25 Gm. $Na_2HPO_4$ in 100 c.c. N/20 NaOH). A final pH of 7.0 is desired. The mixture was centrifuged at 2,500 r.p.m. for forty-five minutes and the clear supernatant fluid removed with a capillary pipette. It is essential that the supernatant be entirely clear.

The extract may be used for both grouping and typing. In our hands, Lancefield's original procedure[7] for grouping extract in which 0.1 per cent glucose broth was used as culture medium gave a better extract for grouping in that larger, more distinct precipitates were formed. It is suggested that this modification be tried in the event of any difficulty encountered in grouping reactions. In carrying out serologic tests on streptococci, reactions should be set up the same day that the extract is prepared. If this is not practicable, prepare the extract with sterile technique. Even slight degrees of bacterial contamination may produce negative or inconclusive results in grouping and typing.

Grouping and typing reactions when carried out in capillary tubes as described by Swift, Wilson, and Lancefield[8] result in a great economy in amount

of serum used. Care must be taken that serum is quite clear, for slight amounts of precipitate may simulate a positive reaction. A small rubber bulb for drawing serum into capillaries is recommended, since the viscosity of the serum makes filling by capillary action slow. A convenient mount for capillary tubes consists of a 150 mm. Petri dish filled one-quarter inch deep with a mixture of one part paraffin and one part petrolatum.

Inconclusive results should be checked by a microprecipitin procedure, using tubes 0.3 mm. in diameter drawn from 6 mm. tubing. Dilutions (1:2, 1:4, 1:8, 1:16) of serum against undiluted extract, and dilutions of extract against undiluted serum, may be necessary to demonstrate specific type in presence of a prozone reaction.

Sera available for grouping and typing in this study are shown in Table I.

Mouse passage was employed to enhance M-substance in fourteen cases. Storage of cultures for four months prior to acquisition of typing serum possibly affected the ability of these strains to elaborate M-substance. Four passages were made, using intraperitoneal injection of 0.05 c.c. of a broth culture and recovering the strain when the mouse was moribund. The peritoneal washings were injected directly into the next mouse. Table III illustrates results of this technique in increasing positive results of typing.

Since sulfadiazine was employed in checking the epidemic, sulfadiazine sensitivity in vitro was determined. To test sulfadiazine sensitivity, strains were grown in clear tryptose semisolid broth containing 0.1 per cent glucose and graded amounts of sulfadiazine. Table II shows results. All strains of Group A were inhibited by a concentration of 0.2 mg. per 100 c.c. of sulfadiazine. The Group H strain was more resistant to sulfadiazine and grew in concentration of 1.0 mg. per 100 c.c. of broth.

TABLE II. SULFADIAZINE SUSCEPTIBILITY OF STREPTOCOCCI STRAINS

| NUMBER OF STRAINS | SULFADIAZINE CONCENTRATIONS IN CULTURE MEDIUM IN MG. PER 100 C.C. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.1 | 0.15 | 0.2 | 0.3 | 0.5 | 1.0 | CONTROL |
| 1 | ++ | − | − | − | − | − | − | +++ |
| 5 | +++ | ++ | − | − | − | − | − | +++ |
| 18 | +++ | ++ | + | − | − | − | − | +++ |
| 2 | +++ | +++ | ++ | − | − | − | − | +++ |
| 1 (Group H) | +++ | +++ | +++ | +++ | +++ | ++ | + | +++ |
| Control (C203MV) | +++ | ++ | + | − | − | − | − | +++ |

+, Barely perceptible clouding of medium at twenty-four hours.
++, Definite clouding of medium at twenty-four hours.
+++, Heavy growth at twenty-four hours.

*Observations.—*

*Morphology:* Three colony types were observed: smooth, mucoid, and rough. Smooth colonies were slightly raised, with indistinct margins and a colony diameter of from 0.5 to 0.7 mm. at twenty-four hours. Mucoid colonies were larger than the smooth colonies, with less distinct margins, a tendency to spread, and a slightly sticky consistency. Rough colonies had undulant margins, a raised dense center, and glistening surface and remained intact when touched with a wire. Smooth and mucoid colonies were evidently matt variants. The

rough colonies tended toward glossy variants, but they still retained matt qualities.

*Serologic Analysis.*—Cultures were grouped at the time of the epidemic but could not be typed until four months later when serum became available. Storage presumably increased the difficulty of obtaining adequate typing reactions due to the loss of M-substance.[9] Table I shows group and type distribution. Twenty-five Group A strains and one Group H were identified. Of the Group A strains, eighteen were type 36, two were type 38, and one was type 3. Four strains which could not be typed failed to kill mice in a dose of 0.1 c.c. of an eighteen-hour broth culture intraperitoneally after four passages. It seems probable that these strains lost virulence and M-substance due to prolonged cultivation.

TABLE III. COLONY MORPHOLOGY, TYPE, AND VIRULENCE RELATIONSHIPS IN GROUP A STRAINS

| COLONY MORPHOLOGY | TYPE | | | | DIRECT TYPING | TYPING AFTER MOUSE PASSAGE (VIRULENT†) | NEGATIVE FOR TYPING AFTER MOUSE PASSAGE (NONVIRULENT‡) |
|---|---|---|---|---|---|---|---|
| | 3 | 36 | 38 | NT* | | | |
| Smooth | | 8(32%) | | | 7(28%) | 1( 4%) | 0 |
| Mucoid | 1(4%) | 1( 4%) | 1(4%) | | 0 | 3(12%) | 0 |
| Rough | | 9(36%) | 1(4%) | 4(16%) | 3(12%) | 7(28%) | 4(16%) |
| Total | 1(4%) | 18(72%) | 2(8%) | 4(16%) | 10(40%) | 11(44%) | 4(16%) |

*No type obtained.

†Mouse killed in twenty-four hours by 0.1 c.c. eighteen-hour broth culture intraperitoneally.

‡Mouse not killed in twenty-four hours by 0.1 c.c. eighteen-hour broth culture intraperitoneally.

Table III shows the relation of colony types and demonstrability of M-substance. Of eight smooth colony strains, seven typed directly and one required mouse passage. Of fourteen rough colony strains, three typed directly. Of the remaining eleven, seven typed after mouse passage. The remaining four strains failed to type at all. Mucoid colony strains required mouse passage before they could be typed. Strains which regained M-substance on mouse passage also showed definite mouse virulence.[8] Increase in virulence to at least ten times the original level was acquired by mouse passage.

## DISCUSSION

A variety of types has been found in studies in streptococcal disease, including scarlet fever and rheumatic fever, as well as in carrier surveys.[10] In this outbreak the predominant organism isolated was the type 36 hemolytic streptococcus. This organism proved to be inhibited in culture by relatively small amounts of sulfadiazine. The clinical outbreak was quickly brought under control with the use of sulfadiazine (1½ Gm. per day to all contacts) combined with an intensive search for, and isolation of, all clinical cases. In addition, the more severe clinical cases responded remarkably well to therapeutic doses of this drug. It is recognized that strains of the same species of organism may vary widely in their susceptibility to sulfonamides. Strains may become resistant to these drugs upon cultivation in media containing minute amounts of sulfonamides, or such strains may be isolated from individuals who have been

treated with amounts of the drug insufficient to bring about the elimination of the parasite from the host. It is reasonable, therefore, to assume that outbreaks of sore throat will vary in their response to the prophylactic administration of sulfadiazine. However, the use of this drug prophylactically in circumscribed outbreaks of streptococcal disease is well justified. Bacteriologically, the types demonstrated lead to the conclusion that type 36 was the predominant causative strain in this outbreak. The presence of additional types, and even of additional groups, of streptococci in an acute outbreak must be taken into consideration. One case exhibited a heavy concentration of Group H streptococcus on initial throat culture. This strain was moderately sulfonamide sensitive and did not kill mice in a dose of 0.1 c.c. of broth culture intraperitoneally. Epidemic outbreaks may be either mixed types or predominantly a single type. Although it is impossible to state categorically that the type 38 or type 3 strain might not have increased in incidence during this epidemic, it is apparent that, at the time the outbreak was checked, the principal inciting agent was *Str. pyogenes*, Group A, type 36.

## CONCLUSIONS

An outbreak of streptococcus pharyngitis demonstrated to be due to Group A hemolytic streptococci was quickly brought under control with sulfadiazine prophylaxis combined with other control measures.

Eighteen type 36, two type 28, one type 3, and four nontypable strains were isolated from the outbreak. Responsible type was most probably the type 36 hemolytic streptococcus.

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# THE SEROLOGIC DIAGNOSIS OF ENDEMIC TYPHUS

## III. The Incidence and Titer of Complement-Fixing Antibodies in Random Samples of the Population in Endemic and Nonendemic Typhus Areas

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IT HAS been recognized for at least a decade that complement-fixing antibodies were demonstrable in the serum of patients who recovered from typhus. Castaneda[1] showed this to be true at least six years after acute infection, and Bengtson and Topping[2] have shown that they persist in most instances for at least several years. In our own experience, it has been found that the reaction occurs in a relatively high titer for variable periods of time.[3]

The specificity of the complement fixation test in endemic typhus, using a rickettsial antigen, was demonstrated by Bengtson and Topping,[2] and the test has been adopted in numerous public health laboratories as a routine diagnostic procedure. In our hands, this has been true for the past two years. Interpretation of the results has been difficult, however, because of the lack of knowledge as to what really constituted a diagnostic reaction and, furthermore, the lack of any information as to the incidence of reactors in the general population. The purpose of this report is to shed some light upon the latter question.

### SOURCE OF BLOOD SPECIMENS

In this study, some 900 sera from bloods collected in the course of a routine serologic survey for syphilis, and representing a random sample of the population, were examined. Five hundred of the specimens were from southern Alabama, Houston County, and represent an area in which typhus is endemic. One-half of these bloods were from persons giving an urban address and one-half from persons with a rural address. Four hundred of the specimens came from residents of northern Alabama, Morgan County, where typhus is of rare occurrence. The residence, whether urban or rural, of these individuals was unknown.

### RESULTS

In summary, the results of complement fixation tests run on these 900 specimens are presented in Table I.

With regard to the distribution of the fifty-eight reactors found in the specimens from Houston County, twenty-three gave an urban and thirty-five a rural residence.

The end titer of a reacting serum was considered to be that dilution in which complement fixation was 3 plus or stronger. On this basis, an analysis of the fifty-nine reacting sera is presented in Table II.

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TABLE I. RESULTS OF COMPLEMENT FIXATION TESTS RUN ON RANDOM SAMPLES OF POPULATION IN ENDEMIC AND NONENDEMIC TYPHUS AREAS

| SOURCE OF SPECIMENS | NUMBER OF SPECIMENS EXAMINED | NUMBER OF SPECIMENS GIVING A COMPLEMENT FIXATION REACTION OF AT LEAST 3 PLUS IN A DILUTION OF 1:4 OR HIGHER |
|---|---|---|
| Morgan County (nonendemic area) | 400 | 1 |
| Houston County (endemic area) | 500 | 58 |

TABLE II. NUMBER OF SPECIMENS GIVING 3 PLUS OR STRONGER REACTION IN A 1:4 OR HIGHER DILUTION OF SERUM

| DILUTION OF SERUM | 1 TO 4 | 1 TO 8 | 1 TO 16 | 1 TO 32 | 1 TO 64 | 1 TO 128 | 1 TO 256 | TOTAL |
|---|---|---|---|---|---|---|---|---|
| Number of sera reacting | 16 | 12 | 13 | 7 | 5 | 4 | 2 | 59 |
| Per cent of sera reacting | 26.9 | 20.3 | 22 | 11.8 | 8.4 | 6.7 | 3.5 | |

## CONCLUSIONS

1. In a section of the United States where typhus is endemic, an examination of sera for complement-fixing antibodies from a random sample of the population shows the incidence of reactors to be high.

2. In a nonendemic area, the incidence of reactors is almost nil.

3. The titer of complement-fixing antibodies detected in presumably normal individuals varied from 1:4 to 1:256.

4. The significance to be attached to a positive complement fixation test for typhus in a febrile patient living in an area of endemic typhus must be weighted by the foregoing observations.

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3. Unpublished data.

# INTERPRETATION OF FINDINGS IN THE CEREBROSPINAL FLUID*

## II. The Technique and Systematic Interpretation of the Albumin-Globulin Ratio in Cerebrospinal Fluids

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NUMEROUS methods have been devised for determining the albumin-globulin ratio in cerebrospinal fluids,[1-11] and considerable importance has been attached to their diagnostic significance. All of the current methods are based on the assumption that the globulin present in cerebrospinal fluid precipitates, as in blood, by 50 per cent saturation with ammonium sulfate. Data presented in this paper indicate that this assumption is true only when considerably higher concentrations of protein are present than in normal cerebrospinal fluids. Some authors believe that even a crude qualitative determination of the ratio might replace the gold reaction. While this was refuted in an earlier paper[12] on the basis of experimental work, it seemed wise to undertake an objective evaluation of the matter. To do this it was necessary to re-examine technical procedures and to establish the general principle of a systematic interpretation by complete syndromes.

### EXPERIMENTAL STUDY

The current methods of determining the albumin-globulin ratio in cerebrospinal fluids[1-11] uniformly use 50 per cent ammonium sulfate as if it were a specific globulin reagent. Actually, it provides a crude separation of albumins and globulins only under very restricted conditions. The following simple experiment may best serve to correct the above misconception.

A mixture of 250 mg. of globulin and 500 mg. of albumin per 100 c.c. was prepared. Its albumin-globulin ratio was 2:1; the protein content, 750 mg. per 100 c.c. The globulin and albumin were prepared from presumably normal human serum, diluted 1:10, by repeated precipitations with 50 and 100 per cent ammonium sulfate, respectively, followed by dialysis and protein determination by micro-Kjeldahl. Various dilutions were prepared with physiologic saline, from 750 mg. per 100 c.c., which corresponds to a serum dilution 1:10, to 15 mg. per 100 c.c., which corresponds to the lowest protein concentration encountered in cerebrospinal fluids. After 50 per cent saturation with ammonium sulfate, followed by centrifuging, the globulin value was determined turbidimetrically. The higher the dilution, the greater the deviation of the albumin-globulin ratio from the known value (2:1); the most marked deviations occurred with protein contents ranging from 15 mg. to about 150 mg. per 100

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*The first paper of this series, stressing the necessity of interpreting every cerebrospinal fluid finding as an integral part of complete syndromes, is number 16 of the references.

c.c. This source of error may lead to incorrect results in routine examinations, since the protein concentration of specimens submitted for study frequently falls in the range mentioned (see Fig. 1).

Theoretically, these results may not be new, but they emphasize the inherent danger in current methods.

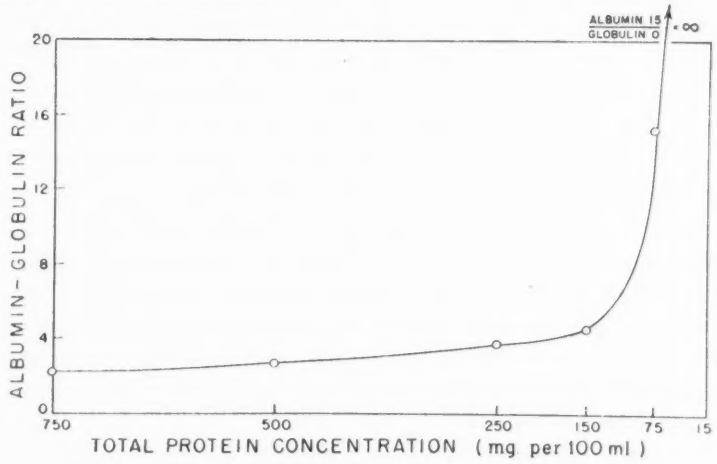


**Fig. 1.—Dilution error using 50 per cent ammonium sulfate.**

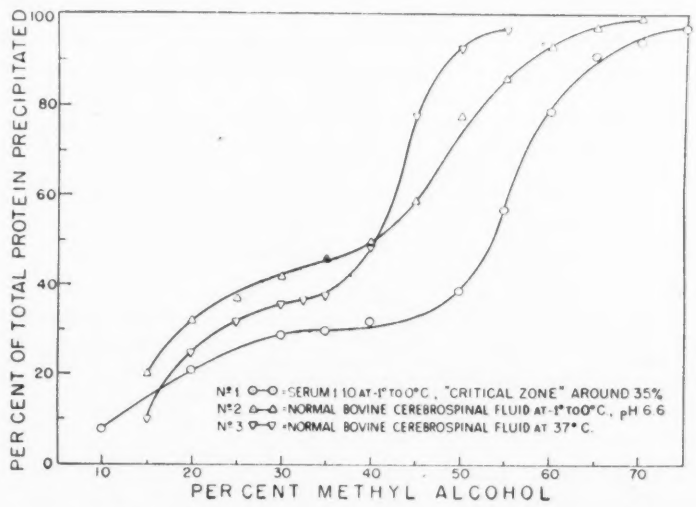


**Fig. 2.—Fractionation curves with methyl alcohol.**

Numerous fractionation curves were determined with serum in higher dilutions. Various salting-out procedures were tried, both in sera and cerebrospinal fluid, and one observation in technique was made that seemed important under the particular conditions of low protein content in cerebrospinal fluids. In salting-out procedures, the high specific gravity of the mixtures prevents collection of the minute amounts of precipitate even when an angle centrifuge[13] is used. When methyl alcohol[14] was substituted for ammonium sulfate, however,

the globulin precipitate was easily collected with an ordinary centrifuge. Thus, it was possible to establish fractionation curves equivalent to those of normal cerebrospinal fluid, while current methods either fail entirely or yield values of questionable significance (see Figs. 1 and 3).

Liu and Wu[14] prefer precipitation at low temperature to avoid denaturation. Denaturation at 37° C., however, proved advantageous in determining the albumin-globulin ratio. Moreover, at low temperature no critical zone was observed, but at 37° C., such a zone, even if indistinct, seemed to lie around 33 per cent. The gold reaction was also used as a check on the optimal separation of globulins and albumins. On the basis of these findings, the use of 33 per cent methyl alcohol at pH 6.6 and 37° C. was adopted.

Fractionation curves of normal human cerebrospinal fluid could not be established because of the large amount (2,000 c.c.) of material required. Bovine cerebrospinal fluid was therefore substituted, since it has been found to have the same protein content and to give the same gold curve as normal human cerebrospinal fluid.[15] Before pooling the bovine cerebrospinal fluids, total protein determinations and colloidal gold tests were made to be sure that only normal fluids were included.

The following study of the albumin-globulin ratio of human cerebrospinal fluid was undertaken. The total protein was determined by micro-Kjeldahl, the percentage of error being from 3 to 5 per cent. From 0.4 to 0.75 mg. of protein was required for reliable results. Cerebrospinal fluids of high protein concentration were diluted to approximately 75 mg. per 100 c.c. or less. The

TABLE I. ALBUMIN-GLOBULIN RATIO IN CEREBROSPINAL FLUID USING METHYL ALCOHOL

| SERIAL NUMBER | CEREBROSPINAL FLUID | TOTAL PROTEIN (MG. PER 100 C.C.) | | ALBUMIN-GLOBULIN RATIO | | | |
|---|---|---|---|---|---|---|---|
| | | CEREBROSPINAL FLUID USED FOR MICRO-KJELDAHL (C.C.) | TOTAL PROTEIN (MG. PER 100 C.C.) | CEREBROSPINAL FLUID USED FOR FRACTIONATION (C.C.) | GLOBULIN (MG. PER 100 C.C.) | ALBUMIN (MG. PER 100 C.C.) | RATIO |
| 1 | Normal | 2 | 25 | 4 | 10 | 17 | 2 |
| 2 | | 2 | 23 | | 7 | 14 | |
| | | | 21 | | 6 | 17 | 2.6 |
| | | | 20 | 4 | 8 | 20 | |
| 3 | Known syphilitic | 2 | 34 | 3 | 11 | 26 | 2.4 |
| 4 | Asymptomatic neurosyphilis | 1 | 60 | 4 | 13 | 50 | 4 |
| 5 | Paretic formula | 0.5 | 132 | 2 | 31 | 97 | 3 |
| | | | 130 | ½* | 28 | 99 | |
| 6 | Acute neurosyphilis (Herxheimer's reaction) | 1 | 85 | 3<br>½ | 7 | 81 | 11 |
| 7 | Tuberculous meningitis | 0.5 | 237 | 3<br>⅓ | 13 | 219 | 17 |
| 8 | Subarachnoid block | 0.5 | 348 | 5<br>⅕ | 11 | 343 | 31 |

*The fractions indicate the dilution of the cerebrospinal fluid that had to be used to reduce its protein content to 75 mg. per 100 c.c. or less.

pH, which becomes markedly alkaline after the fluids have stood, was adjusted by adding one-tenth volume of phosphate buffer of pH 6.6 and about *M*/7 concentration. One volume of methyl alcohol was added to two volumes of buffered cerebrospinal fluid, and the mixture was kept at 37° C. overnight in tightly stoppered centrifuge tubes with pointed bottoms. The amounts of cerebrospinal fluid sufficient for an accurate determination (see Table I) were estimated on the basis of the total protein, the gross appearance, the number of cells, the complement fixation test, and the gold reaction (see Table II). The fractions were separated in ordinary centrifuges, and the values of both globulins and albumins were directly determined, when possible, in duplicate. In general, the protein values checked surprisingly well. After centrifuging, the supernatant was removed, the sediment was washed once with 33 per cent methyl alcohol and then added to the supernatant, and the mixture was precipitated with trichloroacetic acid. To obtain sufficient material within the normal range, a pool of previously tested cerebrospinal fluids was used. In Table I is shown a series of representative cases studied.

The technique described is, of course, inadequate for routine examinations, but it provided a satisfactory standard for the present investigation.

## SYSTEMATIC INTERPRETATION OF THE ALBUMIN-GLOBULIN RATIO

The systematic interpretation[16] of any cerebrospinal fluid finding requires its evaluation as an integral part of a complete syndrome (see Table II). Moreover, a correct interpretation is inconceivable without a satisfactorily standardized technique, which would be difficult to adapt to a routine determination of the albumin-globulin ratio in cerebrospinal fluid.

The current way of interpreting the albumin-globulin ratio may best be illustrated by Fig. 3, copied from Kafka.[4] The globulin values shown by Kafka are relative and not directly comparable with the total protein values; both were obtained by a rather inaccurate volumetric determination, by centrifuging. In the range up to 150 mg. per 100 c.c., the total protein values were calculated in milligrams per 100 c.c. The impression is given in this schematic presentation that the albumin-globulin ratio differentiates, besides the normal, not only syphilis and nonsyphilis, but even three forms of neurosyphilis.

The differences in the albumin-globulin ratio obtained by Kafka[4] in the range from 20 to 150 mg. per 100 c.c. of total protein nearly paralleled the curve obtained when graded dilutions of a mixture of known values were tested (see Fig. 1). Accordingly, there remains only the technically more reliable differentiation between the groups clinically interpreted as "progressive paralysis" and "nonsyphilitic meningitis."

High albumin-globulin ratios are not characteristic of "nonsyphilitic meningitis," such as tuberculous meningitis (see Table I). They are encountered also in acute syphilitic meningitis and in block in the absence of meningitis. Consequently, the problem is to determine the common pathogenetic factor in this etiologically heterogeneous group. To do this, the albumin-globulin ratio must be correlated with the complete syndrome (see Table II). The albumin-globulin ratio invariably is high when fibrin is present. Fibrin is the simplest indicator of a marked increase in the passage of blood proteins into the cerebrospinal fluid,

TABLE II. SYNDROMES OF FIVE GENERALLY OBLIGATORY EXAMINATIONS OF CEREBROSPINAL FLUID (TESTS MADE ON SAME FLUIDS AS IN TABLE I.)

| SERIAL NUMBER | (1) GROSS APPEARANCE | (2) CELLS PER C.MM. | | (3) TOTAL PROTEIN (MG. PER 100 C.C.) | (4) COMPLEMENT FIXATION TEST FOR SYPHILIS | | (5) QUANTITATIVE GOLD REACTION[15]* SERIAL DILUTIONS | | | | | | | | | | EVALUATION OF SYNDROMES | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MONONUCLEARS | POLYMORPHONUCLEARS | | BLOOD | CEREBROSPINAL FLUID | 1:15 | 23 | 34 | 51 | 76 | 114 | 171 | 256 | 384 | 576 | TYPE OF GOLD REACTION | PERMEABILITY AS INDICATED BY SYNDROME | ALBUMIN-GLOBULIN RATIO AS INDICATED BY SYNDROME |
| 1 | Normal | 0/3 | 0 | 25<br>23 | 0 | 0 | 2 | 2.5 | 3.5 | 4 | 4.5 | 4 | 3 | 2.5 | 2 | 1.5 | Negative | Normal | |
| 2 | Normal | 1/3 | 0 | 21<br>20 | 0 | 0 | 2 | 2.5 | 3.5 | 4.5 | 4 | 3.5 | 3 | 2 | 1 | 1 | Negative | Normal | |
| 3 | Normal | 8/3 | 0 | 34 | + | 0 | 4.5 | 5 | 5.5 | 6 | 6.5 | 5 | 4.5 | 4 | 2 | 1.5 | Negative | Normal | Low |
| 4 | Normal | 22/3 | 0 | 60 | + | + | 6 | 6.5 | 7 | 7.5 | 8 | 9 | 7 | 6 | 5 | 3.5 | So-called syphilitic | Normal | |
| 5 | Normal | 186/3 | 2/3 | 132<br>130 | + | + | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | So-called paretic | Inconsiderably increased | |
| 6 | Slender fibrin coagulum | 123/3 | 56/3 | 85 | + | + | 4 | 5.5 | 6.5 | 7.5 | 8.5 | 11 | 13 | 11 | 9 | 6.5 | So-called meningitic | Markedly increased | |
| 7 | Cobweblike fibrin coagulum† | 287/3 | 74/3 | 237 | 0 | 0 | 1.5 | 2 | 2.5 | 3.5 | 4 | 6 | 7 | 8.5 | 8 | 7.5 | So-called meningitic | Highly increased | High |
| 8 | Spherical fibrin coagulum‡ | 5/3 | 0 | 348 | 0 | 0 | 0.5 | 1.5 | 3 | 4.5 | 7 | 9.5 | 11 | 13 | 15 | 12 | So-called meningitic | Highly increased | |

*The color values are determined with the gold color standard: 0, red; 1 to 9, blue-red; 10, blue; 11 to 19, blue-white; 20, colorless, complete coagulation. ∧ indicates the location of the maximum reaction.

†The syndrome provides the "special indication," in addition to the "generally obligatory five examinations," to examine for tubercle bacilli, which were demonstrated.

‡The syndrome of block provides the "special indication" to examine cerebrospinal fluid collected by cisternal puncture, in which the secondary effects of block are absent. In this case the cerebrospinal fluid was colorless.

or, in other words, of permeability of the meninges. A second criterion of importance is the presence of polymorphonuclear leucocytes in (sub-)acute inflammatory processes. A third, more universal, indicator of the state of permeability is furnished by the gold reaction. When fibrin or abundant polymorphonuclear leucocytes indicate markedly increased permeability, the gold reaction invariably yields the so-called meningitic or rather hematogenous type of curve (see Table II; Nos. 6 to 8). The paretic or parenchymatous type (see Table II, No. 5) indicates no or little increase in permeability, while the syphilitic and negative types (Table II, No. 4, and Nos. 1 to 3) indicate normal permeability.* These three criteria make the use of other special tests for permeability unnecessary.

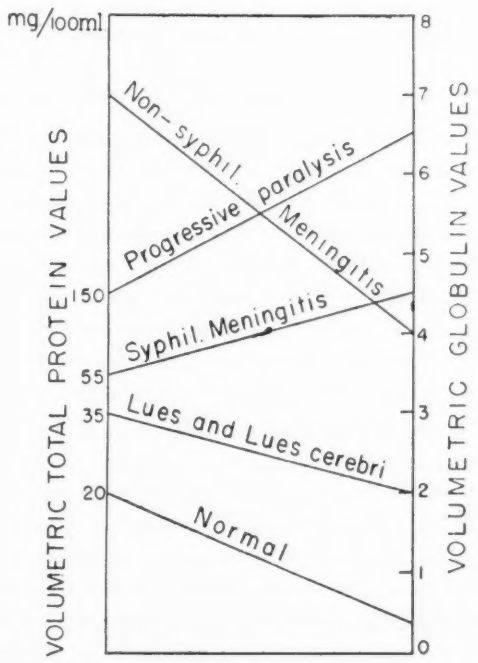


Fig. 3.—Schematic presentation of albumin-globulin ratios from Kafka.[4]

It is, of course, self-evident that the albumin-globulin ratio in cerebrospinal fluids does not depend entirely on the presence of blood proteins. Under conditions of normal permeability, the albumin-globulin ratio is affected by the intrameningeal (nonhematogenous) production of protein. The determination of the albumin-globulin ratio, however, is not necessary in routine examinations. High albumin-globulin ratios merely confirm data already provided by other means. Low ratios are encountered in normal conditions and in chronic neurosyphilis. The values obtained with chemical methods, however, are technically so unreliable and so overlapping that they are seldom characteristic for any group.

*These designations have been widely adopted; however, since the terminology may be misleading, it is recommended that the curves be designated as A, B, C, and D: that is, findings within normal limits should be classified as A; those giving evidence of chronic productive inflammatory changes, as B; hematogenous types of curve, as C; and those showing evidence of parenchymatous degeneration, as D.

More correct values of albumin-globulin ratios in cerebrospinal fluids were obtained by Kabat and associates[17] with electrophoresis. Unfortunately, this method cannot be used routinely, because it requires about 70 c.c. of cerebrospinal fluid. However, it opens new possibilities in diagnosis that seem to lie not in the determination of albumin-globulin ratios but in the determination of the single protein fractions. Present indications are that these fractions in cerebrospinal fluids can be determined with specific antisera. This procedure would be economical since no fractionation would be required. Experiments are now in progress with two fractions, albumin and gamma globulin. The albumin-globulin ratio can be determined in cerebrospinal fluids by the method used for sera by Goettsch and Kendall.[18] The determination of gamma globulin with values similar to those found by Kabat seems, however, to promise more significant progress in the diagnostic examination of cerebrospinal fluid.

## SUMMARY AND CONCLUSIONS

Current methods of determining the albumin-globulin ratio in cerebrospinal fluid are markedly inferior to those for blood. The albumin-globulin ratio in cerebrospinal fluid varies widely because of the amount of blood proteins that may be present. Even crude qualitative methods can demonstrate some differences, provided the protein content is high. In low protein concentrations, which prevail in neurosyphilis, misleading results are obtained by using 50 per cent saturation with ammonium sulfate to precipitate the globulin. With methyl alcohol, it is possible to secure consistent results in normal cerebrospinal fluids and to determine albumin-globulin ratios in various pathologic conditions.

Evaluation of the albumin-globulin ratio should, however, be based on a systematic interpretation of the results of various laboratory tests. An attempt to correlate an isolated finding directly with clinical conditions is, as a rule, misleading. It should be interpreted as an integral part of a complete syndrome. The application of this basic principle indicates that determination of the albumin-globulin ratio in cerebrospinal fluids provides, chiefly, information regarding the permeability of the meninges, which is more conclusively indicated by other procedures.

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# THE INFLUENCE OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL) UPON NORMAL PERSONS AND UPON THOSE SUFFERING FROM DISTURBANCES OF THE AUTONOMIC NERVOUS SYSTEM

## Preliminary Report

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DIMETHYLAMINOETHYL benzhydryl ether hydrochloride, an alkamine benzhydryl ether, the structural formula of which is given, has been shown to possess antispasmodic and antihistamine activity of high degree.[1-4]

Chemically, this drug fails to resemble other agents employed clinically because of similar properties. Moreover, the present studies suggest that it lacks some of the unpleasant and at times harmful effects of such drugs. For these reasons, it has seemed important to observe the action of the drug in a relatively wide range of dosage upon the various functions and systems of the body, both in normal subjects and in those suffering from widely diverse illnesses.

### Materials and Methods

Twenty-eight women from 25 to 65 years old, with an average age of 40.2 years, and thirty-two men from 21 to 60 years old, with an average age of 43.3 years, have served as the subjects of these tests. These subjects were divided into two major groups: (1) those who had no known disease or at least no condition in which the autonomic nervous system was directly involved, and (2) those known to have some disturbance in which the autonomic nervous system played a role. The former group consisted of sixteen women and eighteen men. Of these thirty-four subjects, eight (three women and five men) were given the complete cycle of examinations before, during the time, and after they had received dimethylaminoethyl benzhydryl ether hydrochloride, hereinafter referred to as Benadryl.* Two subjects were used to determine the effect of the drug on sleep only. Ten were used to determine the intravenous effects and blood levels only, and fourteen were chosen for the study of the local action of the drug upon the eye.

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*Generous supplies of this material under the trade name Benadryl were made available through the courtesy of Dr. E. A. Sharp, Director of Clinical Investigation, Parke, Davis & Company, Detroit, Mich.

TABLE I. EFFECTS OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE IN CERTAIN CLINICAL CONDITIONS

| DIAGNOSIS | NUMBER OF PATIENTS | CHANGE IN CLINICAL CONDITION OBSERVED UNDER TREATMENT | | | REMARKS |
|---|---|---|---|---|---|
| | | COMPLETE RELIEF | IMPROVED | UNCHANGED | |
| Spastic colon | 7 | 3 | 4 | 0 | In two cases, period of time may be too short for final conclusion |
| Cardiospasm | 2 | 0 | 0 | 2 | |
| Other gastrointestinal neuroses | 5 | 3 | 0 | 2 | Chief symptoms involved were nausea, vomiting, and pain |
| Essential hypertension | 1 | 0 | 1 | 0 | Drop in systolic and diastolic blood pressure and disappearance of headache |
| Hyperthyroidism | 2 | 0 | 0 | 2 | Thyroid condition controlled prior to amine treatment; constipation and exophthalmos not relieved |
| Paget's disease with paralysis agitans | 1 | 0 | 1 | 0 | Tremor alone improved |
| Climacterium | 1 | 0 | 1 | 0 | Abdominal pains completely relieved |
| Dysmenorrhea, functional | 2 | 2 | 0 | 0 | Syndrome of "premenstrual tension" |
| Asthma | 4 | 4 | 0 | 0 | One initially seen in status asthmaticus |
| Allergy to insulin, local | 1 | 1 | 0 | 0 | |
| Totals | 26 | 13 | 7 | 6 | |

The major diagnosis encountered in each of the second group of patients is listed in Table I. In each patient of this group, there was reason to believe that a disturbance of the autonomic nervous system existed.

Details of histories, physical examinations, and laboratory tests have all been designed to afford a thorough appraisal of the status of important bodily functions and are mentioned under Results.

Unless otherwise mentioned, in "diseased" individuals 1 capsule (50 mg.) of benadryl was given three times daily.

For the eight subjects in the control group, diet was roughly regulated according to weight (Table II).

Repetition of the initial observations on these eight subjects was made at intervals of from one to two weeks before, during, and after oral administration of the drug over total periods of time ranging from six to eighteen weeks. In two of these eight control subjects, intravenous tests were performed for the purpose of observing blood levels during the immediately succeeding hours. In eight other subjects, similar observations were made following intravenous therapy.

TABLE II

| WEIGHT (POUNDS) | DIET (GM.) | | | CALORIES |
|---|---|---|---|---|
| | CHO | PROTEIN | FAT | |
| 140 or less | 135 | 70 | 80 | 1,540 |
| 140 or 175 | 175 | 75 | 100 | 1,900 |
| 175 or more | 200 | 95 | 125 | 2,300 |

Patients in whom the findings are recorded received the drug for periods of from two to eighteen weeks.

## RESULTS

For purposes of clarity and comparison, data from control subjects and patients are summarized together, except in those instances where the latter differed materially from the former. In view of the nature of the study, the data are arranged under the systems of the body in so far as possible.

*General Metabolism.*—No alterations in weight or basal metabolic rate of either patients or control subjects could be ascribed to the direct effects of administered benadryl.

*The Central Nervous System.*—Both normal and "sick" subjects became more placid while receiving treatment with benadryl.

Seven of ten "normal" subjects observed that they slept better than formerly. Two of these seven subjects observed sleepiness during the day as a result of the ingestion of a single 50 mg. capsule. Among the twenty-six patients who received the drug (Table I), sleep was improved in seventeen and was unchanged in five; the status of sleep was not recorded in four subjects. A tendency toward daytime somnolence was observed by three patients. One of these had to reduce the dose of drug from 150 to 100 mg. but was later able to resume the previous dose without further unpleasant symptoms.

*The Eye.*—No variations in the size of the pupils under fixed conditions of lighting were observed in nine patients before and after therapy with orally administered benadryl, or during its use in daily doses ranging from 150 to 400 mg., even after the largest doses were employed for five weeks at a time.

Locally, the effects were quite different. An aqueous solution (4 per cent) was well tolerated by the conjunctiva of rabbits, but a stronger solution (5 per cent) caused hyperemia and edema. For human experiments, we used 0.5 and 1.0 per cent solutions of the drug without discomfort to the patients unless the solution had stood in a clear bottle for several weeks, after which it became irritative.

Most of the work was done with the lesser concentration. This caused an average pupillary dilatation of 1.0 mm. within fifteen minutes in nine of fourteen and of 1.8 mm. in one hour in all of the fourteen patients. An interference with accommodation always accompanied the mydriatic effect. Epinephrine hydrochloride (1 per cent solution) and benadryl (0.5 per cent solution) were applied simultaneously in six patients. Within fifteen minutes all but one patient showed a dilatation averaging slightly less than 1.0 mm. and in one hour an average dilatation amounting to 1.7 mm.; this was practically identical with that produced by the solution of the alkamine ether alone.

In fifteen trials on fourteen patients this amount of epinephrine, without the benzhydryl ether, showed no appreciable reaction in the size of the pupil even after one hour. We may conclude, therefore, that epinephrine hastened the action of the drug but did not alter its total effect.

Homatropine sulfate (1 per cent solution) and eserine sulfate (1 per cent solution) produced their characteristic mydriatic and miotic effects, respectively.

These were not influenced by the use of benadryl administration orally in doses up to 400 mg. daily in any one of the five patients tested.

*The Respiratory System.*—Alterations in the rate and character of the respirations were not observed in any but asthmatic individuals. In these subjects, administration of benadryl caused a decrease in respiratory rate and a rise in vital capacity to normal levels (Table III). Vital capacity was determined in all the control subjects and in two of the four patients with asthma, using the Benedict-Roth respirometer and the Dreyer formula.[5]

TABLE III. RESPIRATORY RATE AND VITAL CAPACITY IN SUBJECTS TAKING DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

| SUBJECTS | DOSAGE (MG. PER DAY) | DURATION OF TREATMENT (WEEKS) | VITAL CAPACITY (PER CENT) | RESPIRATIONS (PER MINUTE) |
|---|---|---|---|---|
| Eight controls | 0 | -- | 90 | 16 |
| | 150 | 4 | 101 | 18 |
| | 300 | 3 | 89 | 17 |
| | 400 | 4 | 97 | 18 |
| Two with asthma | 0 | -- | 40 | 28 |
| | 150 | 1 | 43 | 26 |
| | 300 | 3 | 85 | 18 |
| | 400 | 5 | 84 | 17 |

*The Cardiovascular System.*—Following the oral administration of the alkamine ether, *dizziness and blurring of vision* were the only symptoms suggesting cardiovascular disturbances which could have been attributed with reasonable certainty to the drug. Even though 100 mg. were given four times daily, these symptoms were not seen in any subjects of the control group; however, they were observed in two patients with a gastrointestinal neurosis and in one patient with dysmenorrhea. Irregularities in the circulation, rather than a direct toxic action upon nervous tissue, were thought to be responsible, since two of the three patients showed an associated orthostatic hypotension (see paragraph on blood pressure). On the other hand, orthostatic hypotension did not necessarily produce dizziness or blurring of vision, since six subjects developed this condition without any associated symptoms.

The *size of the heart and of the liver* showed no clinically detectable alteration even after prolonged treatment (up to eighteen weeks) with as much as 100 mg. of dimethylaminoethyl benzhydryl ether hydrochloride four times daily by mouth. Teleroentgenograms of the heart and lungs of all subjects revealed no changes due to the action of the drug.

In thirty-six subjects given 300 mg. or less of the drug daily, no alterations in the *pulse rate* were observed, except in the group of patients with asthma.

Lowering of the systolic *blood pressure* was observed in three of forty-six subjects in whom serial examinations were made. In two of these patients who were taking 150 mg. daily, dizziness of mild degree was noted. The third subject was given 200 mg. daily without untoward subjective symptoms; the average degree of depression was 15 mm. of mercury. Orthostatic hypotension was observed in a total of six subjects. Three of the six were control subjects who developed the condition while taking 300 mg. or more daily. One patient with

cardiospasm and one with dysmenorrhea, each receiving 300 mg. daily, and one individual suffering from hyperthyroidism and dyschesic constipation, who was given 200 mg. daily, completed the group in which orthostatic hypotension was observed. In all but one individual, these changes in the blood pressure reverted to normal within several days after discontinuing the drug. This individual was a control subject who had received 400 mg. of the alkamine ether daily for approximately three and one-half weeks and had previously taken smaller doses over a total period of five weeks. Two months after cessation of therapy, an orthostatic hypotension persisted which may not have been due to the drug, since pressures were not checked in all positions prior to beginning its administration.

After the oral administration of as much as 400 mg. of benadryl daily, no appreciable changes could be demonstrated in the *capillary loops* of the fingernail beds of the five "normal" subjects and of the one asthmatic patient studied.

The *Rumpel-Leede phenomenon* was routinely negative in subjects using the drug.

The *circulation times and capillary permeability* were determined in six subjects by the fluorescein technique of Lange and Boyd.[6] Circulation times were apparently not influenced by the drug in doses up to 400 mg. daily by mouth (Table IV). Capillary permeability was decreased by large doses of the drug (Table IV).

TABLE IV. DETERMINATION OF CAPILLARY PERMEABILITY AND CIRCULATION TIME BY FLUORESCEIN TECHNIQUE IN EIGHT CONTROL SUBJECTS BEFORE, DURING, AND AFTER ADMINISTRATION OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

| NUMBER OF SUBJECTS | DOSAGE (MG. PER DAY) | DURATION TREATMENT (WEEKS) | C. T.* (SEC.) | | CAPILLARY PERMEABILITY (PER CENT NORMAL)† | |
|---|---|---|---|---|---|---|
| | | | RANGE | AV. | RANGE | AV. |
| 8 | 0 | -- | 27 to 42 | 33 | + 2 to -21 | -11 |
| 8 | 150 | 4.3 | 28 to 36 | 30 | + 6 to -39 | -20 |
| 2 | 200 | 3.0 | 26 to 46 | 36 | - 4 to -20 | - 9 |
| 6 | 300 | 3.0 | 28 to 36 | 33 | -24 to -41 | -33 |
| 4 | 400 | 3.5 | 28 to 44 | 38 | -24 to -61 | -38 |

*Arm to leg.

†Calculated from the average mean curve. Variations of plus or minus 25 per cent are still within the normal range.

*Electrocardiographic tracings* showed no changes as a result of the use of the alkamine ether.

When a solution of *dimethylaminoethyl benzhydryl ether hydrochloride* was given *intravenously*, three of ten subjects developed "reactions." One normal subject had taken the drug orally in divided daily doses of 150 mg. for one week, 300 mg. daily for two weeks, and 400 mg. daily for four weeks, without ill effects. Four hours after an additional dose of 20 mg. given intravenously, this subject complained of weakness, headache, "seasickness," and drowsiness, all associated with extreme pallor, but no sweating. Despite the continuance of the drug orally, all these symptoms had disappeared by the following day. A second subject of the "normal" group had completed three months of treatment with oral doses up to 400 mg. of the amine daily. Eleven days later 30

mg. were given intravenously. Fifteen minutes thereafter, the subject complained of dizziness with a sensation of falling toward the left. The entire reaction disappeared in one hour without treatment. The third reaction to the intravenous injection of the alkamine ether occurred in a patient with controlled thyrotoxicosis who had not previously received the drug. Almost immediately after a dose of 30 mg., the patient developed a severe chill associated with sweating, headache, low back pain, and a slight decrease in temperature (to 97.6° F.) but with no change in either pulse rate or blood pressure. The remaining seven subjects, to none of whom had the drug been administered previously, showed no untoward reaction of any kind to the intravenous injection of as much as 30 mg. Such doses appreciably altered neither the respiratory rate nor the cardiac function, nor did they produce any changes either in degree of perspiration or in the color of the skin.

*The Gastrointestinal System.*—No gross change was observed in the quality or quantity of *salivary secretion* in any of the eight control subjects when as much as 400 mg. of the benzhydryl ether were taken daily. In one patient with gastrointestinal neurosis, in whom 150 mg. of the drug were given daily, increased thirst and dryness of the mouth were subjectively noted, but a second patient with similar diagnosis and treatment experienced mild salivation.

*Mouth-anus time* as determined in six control subjects through the ingestion of charcoal (1 Gm.) was not altered by the drug. *Bowel habits* were unchanged by the drug in all control subjects and in all the patients except the seven who suffered from a spastic colon. Follow-up barium enemas revealed a decrease in the spastic condition in the four patients with spastic colon in whom such an examination was possible.

Seventeen of the twenty-six patients complained of abdominal pain when first seen (Table V). Under treatment with the drug, ten of the seventeen showed partial or complete relief of such distress. In two of the remaining seven patients, sufficient time has not elapsed to draw final conclusions, although usually the drug acts quickly. The pain of three patients was not relieved. Details are shown in Table V.

In regard to *gastric acidity,* there was a marked decrease in the amount of both free and combined acid as a result of the administration of the benzhydryl ether in four of five control subjects who showed free acid prior to the use of benadryl. Moreover, there was little or no response to histamine. The details

TABLE V. VARIATIONS IN ABDOMINAL PAIN IN PATIENTS TREATED WITH DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

| DIAGNOSIS | NUMBER OF PATIENTS | PAIN | | | FOLLOW-UP INSUFFICIENT |
|---|---|---|---|---|---|
| | | COMPLETE RELIEF | IMPROVED | NO CHANGE | |
| Spastic colon | 7 | 4 | 1 | 2 | 0 |
| Cardiospasm | 2 | 0 | 0 | 2 | 0 |
| Other gastrointestinal neuroses | 5 | 1 | 1 | 1 | 2 |
| Climacterium with reflex gastrointestinal disturbances | 1 | 0 | 1 | 0 | 0 |
| Dysmenorrhea, functional | 2 | 2 | 0 | 0 | 0 |

are included in Figs. 1 to 4. The fifth patient showed a definite drop in the acid values, but the dosage and duration of treatment were not sufficient to develop the full effects of the drug.

Three of eight patients with gastrointestinal disturbances, upon whom fractional gastric analyses were performed, showed an anacidity except after histamine stimulation, presumptively as a result of old age. The acid values following histamine injection were markedly decreased in all of these by the

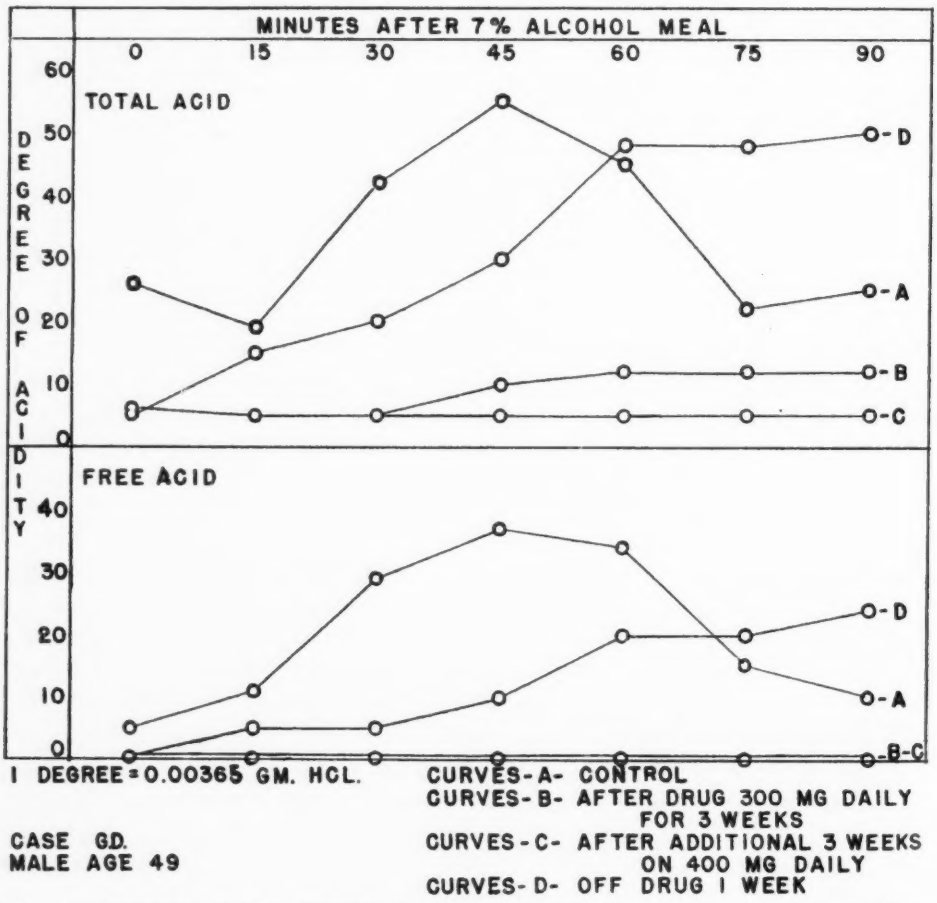


Fig. 1.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

use of the benzhydryl ether in doses of from 150 to 200 mg. daily for periods of two, three, and four weeks, respectively. The other five patients exhibited responses in the gastric secretion similar to those observed in the control subjects.

*Renal Function.*—No variation in urinary volume was observed as a result of the use of dimethylaminoethyl benzhydryl ether hydrochloride, either in the control subjects or in patients. In six subjects, Fishberg's modification of Volhard's concentration and dilution tests[7] failed to show any variations from the

normal. Repeated routine urinalyses were likewise uninformative. Urea nitrogen and nonprotein nitrogen determinations, performed upon the blood of all subjects before, during, and after treatment, remained consistently within normal limits. No indication for further investigation of renal function arose during the course of the experiments.

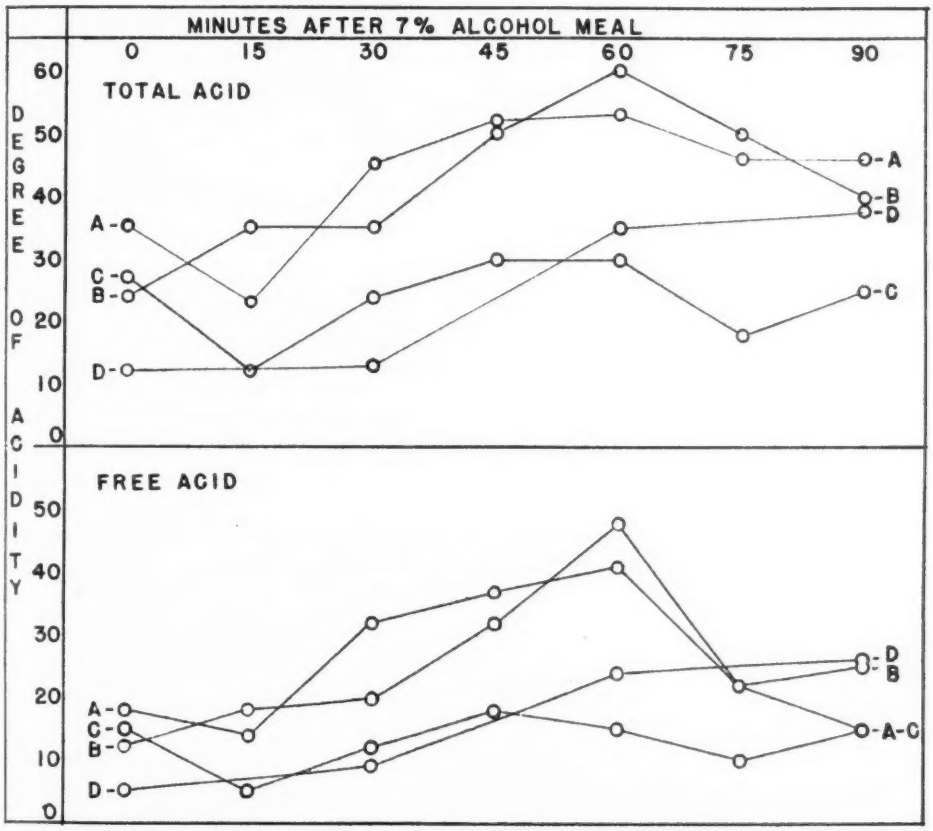


Fig. 2.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

*Skin.*—Following the oral, intravenous, and topical application of the benzhydryl ether, there was no demonstrable alteration in skin color or temperature, but definite influence upon capillary permeability (see page 564) was seen and the histamine-wheal reaction was strikingly altered. Dimethylaminoethyl benzhydryl ether hydrochloride was applied to the volar surface of the forearm as a 1 per cent ointment in a cholesterol base, using first an aqueous

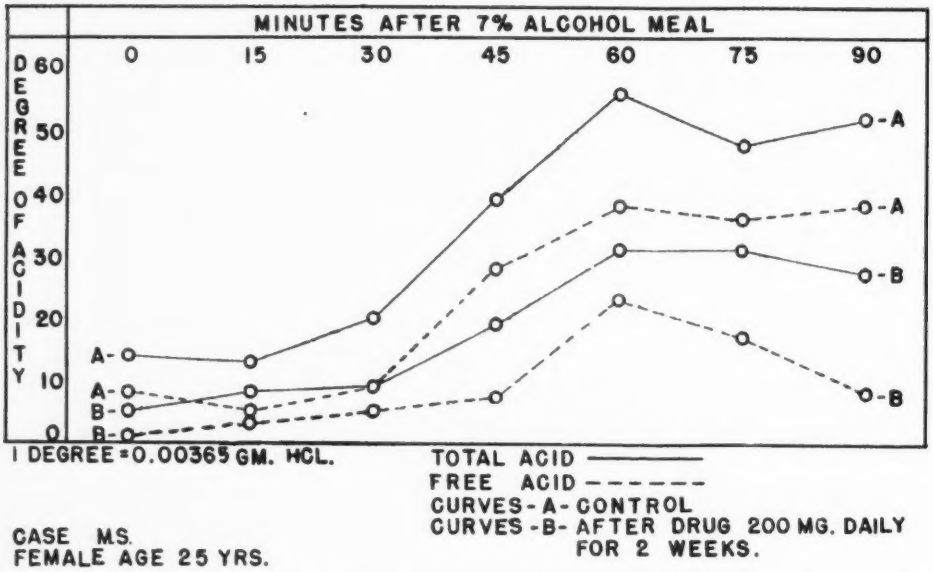


Fig. 3.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

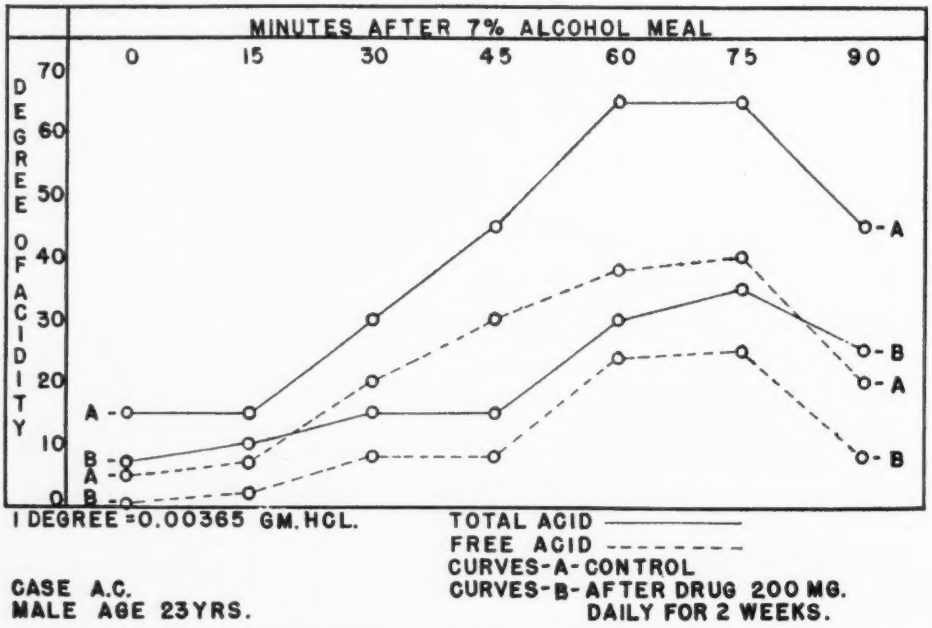


Fig. 4.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

and later a nonaqueous phase. One hour later histamine was applied in the usual way to both forearms. In no instance was there a difference in the size or intensity of the histamine wheal and erythema as a result of the previously applied ointment. However, a similarly prepared ointment of 5 per cent strength completely suppressed the response to histamine.

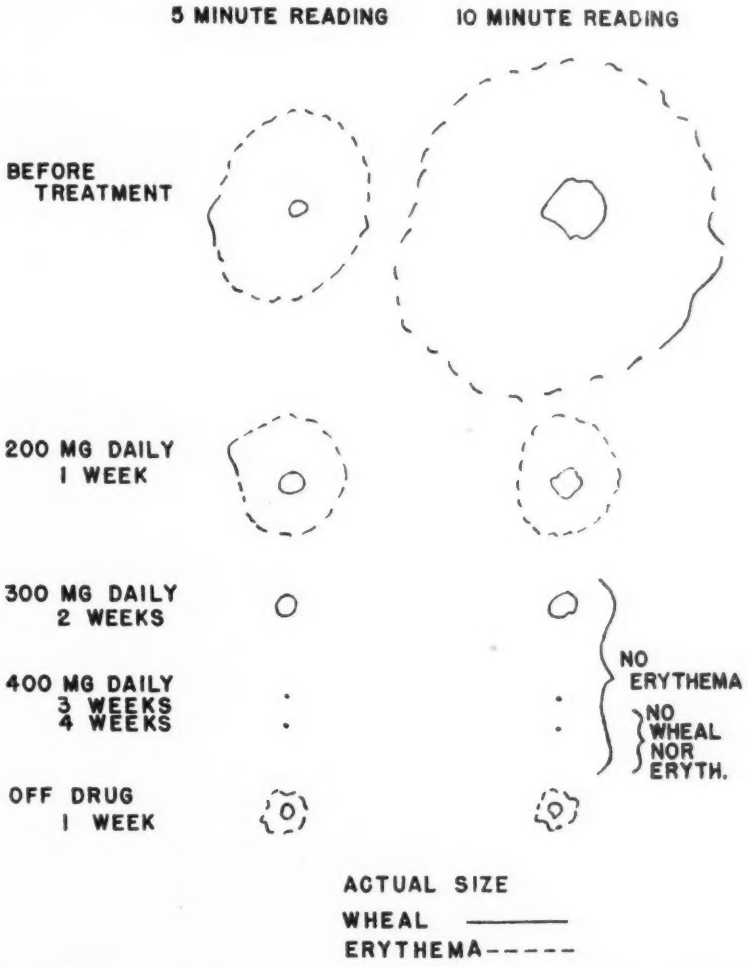


Fig. 5.—Dermal reaction to histamine, five and ten minutes after application, respectively, in patient (O. K.) before, during, and after treatment with dimethylaminoethyl benzhydryl ether hydrochloride (two-thirds actual size).

The average results of skin sensitivity to histamine applied intradermally in five control subjects according to a method previously described[8] are noted in Figs. 5 to 7. The degree of reaction in each instance is recorded as a percentage of the initial response, or response of the "fore" period. As was to be expected, sensitivity to histamine varied considerably from patient to patient. For instance, in two subjects the sensitivity was not sufficiently great prior to treatment with the drug to justify continuation of study in them. Because of

these facts, each of the five subjects mentioned was made his own control. It is clear from Fig. 5 that the average therapeutic dose of 50 mg. of the benzhydryl ether administered three times daily, was capable of producing an appreciable reduction in the severity of the histamine reaction. A further striking decrease occurred when 300 mg. per day were given, beyond which additional increments in dosage had little, if any greater, effect (up to 400 mg. daily tried).

These skin reactions closely paralleled the behavior of the systemic reactions to the histamine injections which were used in carrying out the fractional

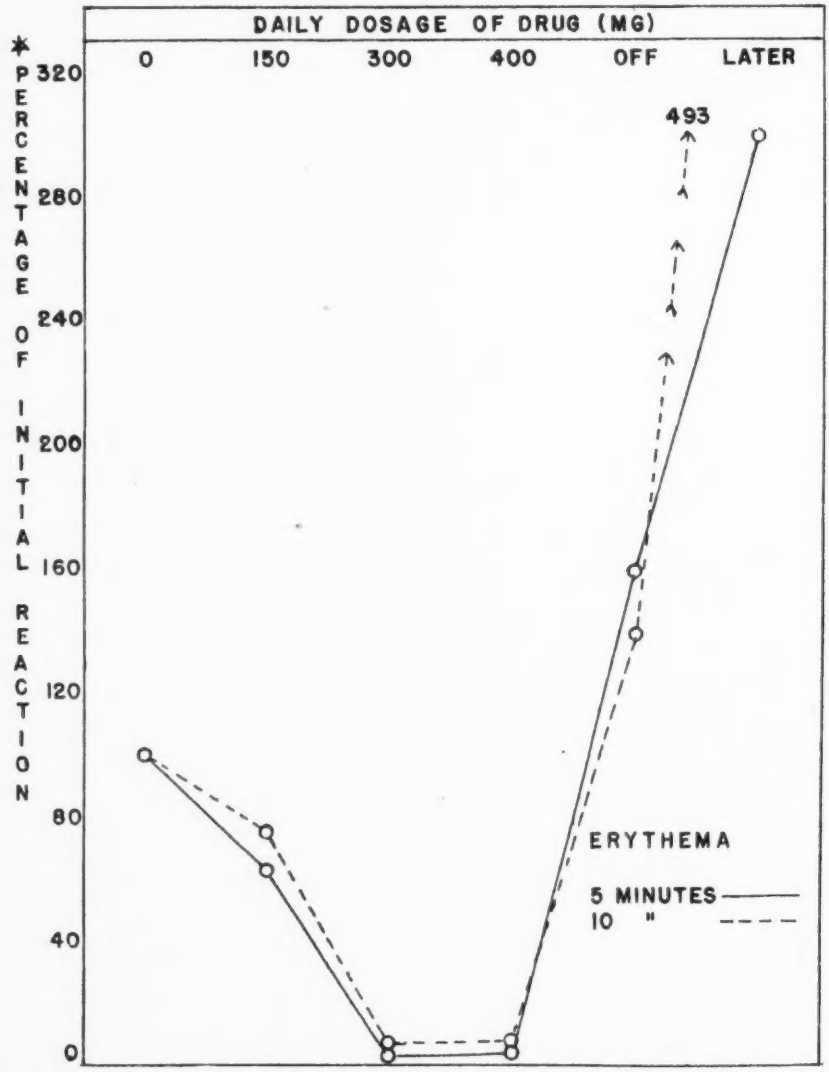


Fig. 6.—Influence of varying dosage of dimethylaminoethyl benzhydryl ether hydrochloride upon skin sensitivity to histamine (average results in five subjects).

*The degree of reaction in the "fore" period has been placed at 100 for each subject and subsequent values were derived as a percentage of this value.

gastric analyses (0.25 mg.). Two "normal" subjects obtained such a reaction in the "fore" period, which was completely abolished by the use of 150 mg. of the drug daily for as little as ten days. Reactions were ameliorated in one asthmatic patient by the use of 300 mg. of the drug and completely abolished in the same patient by 400 mg. daily.

*Blood.*—The erythrocyte and leucocyte counts, hemoglobin determinations, and differential white counts performed in all subjects before and in most of them during treatment with dimethylaminoethyl benzhydryl ether hydrochloride failed to show any significant changes. Hematocrit readings and the speed of sedimentation of the erythrocytes noted in eight control subjects and in four patients were comparable in each individual instance before, during, and after treatment.

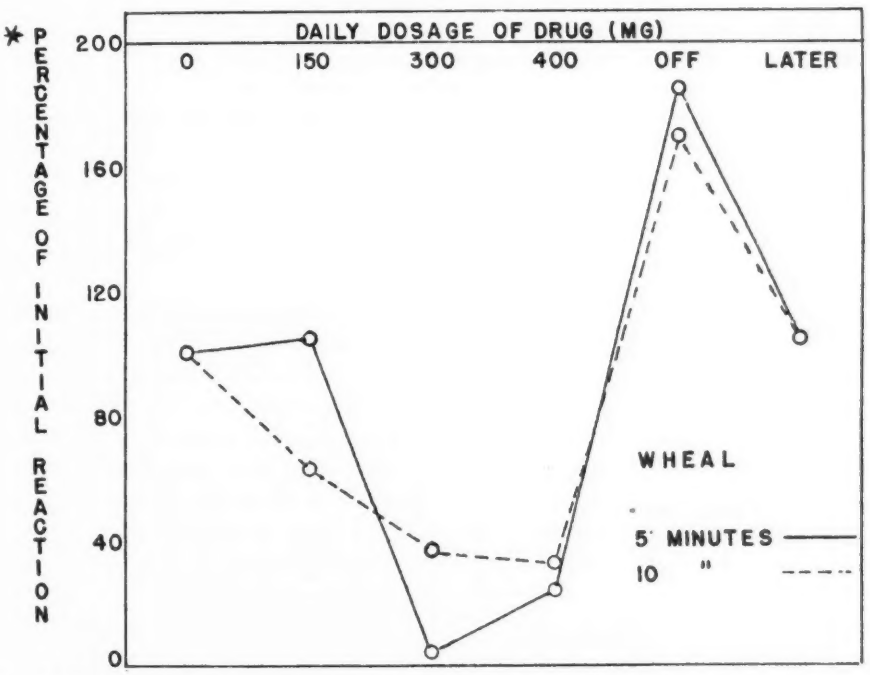


Fig. 7.—Influence of varying dosage of dimethylaminoethyl benzhydryl ether hydrochloride upon skin sensitivity to histamine (average results in five subjects).
*See Fig. 6 for information.

In all sixty subjects, the blood nonprotein nitrogen, urea nitrogen, creatinine, and glucose were within normal range before, during and after therapy with the alkamine ether. Total proteins, the albumin-globulin ratio, total and free cholesterol, alkaline phosphatase, the icteric index, and the van den Bergh reaction, and the cephalin flocculation were determined in sixteen subjects by a method previously described.[9] No significant variations occurred in relation to treatment with benadryl.

*The Therapeutic Activity of Dimethylaminoethyl Benzhydryl Ether Hydrochloride.*—The responses of twenty-six patients who have received dimethyl-

aminoethyl benzhydryl ether hydrochloride are tabulated in Table I. These suggest the probable future trends of therapy with benadryl.

*Untoward Reactions.*—An undue sense of *fatigue and sleepiness* occurred in approximately 15 per cent of thirty-six subjects. *Dizziness and blurring of vision* was noted in three subjects. A transient partial amnesia occurred in one subject who received 400 mg. of the drug in a single dose.

## DISCUSSION

*Untoward Reactions.*—In the acceptance or rejection of any agent for therapeutic purposes, the nature, severity, and duration of untoward reactions must be prime considerations. The unpleasant symptoms produced by dimethylaminoethyl benzhydryl ether hydrochloride have been of mild degree, sometimes disappearing while the medication was continued. They have always been relieved promptly by discontinuing the drug or by decreasing the amounts administered. Indeed, when the amine is given orally, their occurrence is extremely rare. Other workers have had a similar experience.

*Nature of the Action of Benadryl.*—The vigorous antihistamine action of a series of benzhydryl alkamine ethers, to which dimethylaminoethyl benzhydryl ether hydrochloride belongs, has been amply demonstrated in animals[1, 2, 4] and in human beings.[3] The present studies further illustrate the clinical applicability of these effects, since patients with asthma, functional dysmenorrhea, and urticaria have been successfully treated.

The role of histamine in the production of functional dysmenorrhea may be questioned, but the relation of certain types of this condition to the activity of the autonomic nervous system seems to be rather clear.[10-13] The influence of dimethylaminoethyl benzhydryl ether hydrochloride upon the clinical syndrome of spastic colon is not so clear, although here again a direct antihistamine effect may be operative,[14] particularly in view of the fact that histamine may be readily synthesized by the intestine.[15] Further evidence for the antihistamine nature of the action of the drug is its ability to lower gastric acidity. Current concepts accord histamine a normal role in the production of hydrochloric acid by the stomach.[14, 16, 17] It seems logical to assume from the present data that the decrease or even disappearance of free hydrochloric acid from the gastric juice of patients receiving the alkamine ether is due to its retarding effect upon that activity of histamine.

The tendency for histamine to cause tissue exudation may be related to an alteration in the permeability of the capillaries. If that is so, then the decrease in permeability produced by the benzhydryl ether in the present series of experiments would be additional proof of an antagonism to histamine.

When the antispasmodic action of dimethylaminoethyl benzhydryl ether hydrochloride is compared with that of papaverine, its potency is "approximately 650, 50, and 1.3 times that of the latter drug relative to antagonism of histamine, acethylcholine, and barium chloride, respectively."[4] Therefore, while the antihistamine action is by far the most striking feature of the action of dimethylaminoethyl benzhydryl ether hydrochloride, its direct effect upon the autonomic nervous system deserves consideration.

Topical application of the drug to the eye results in pupillary dilatation. This appears to be an atropine-like or at least a parasympathetic depressant, effect. The response is hastened but not increased by the simultaneous use of epinephrine.

The failure of the drug to accelerate the pulse, except in the largest doses, or to increase blood pressure is further evidence that the direct sympathomimetic influence is slight or nonexistent, while the antihistamine effect is great. This makes the drug particularly desirable as an antispasmodic in asthma, where too often cardiac acceleration and even failure exist before treatment is begun. The mild quieting action of the drug is of value in connection with all of the gastrointestinal neuroses, the urticarias and, indeed, any allergic condition; such sedative property is not shared by epinephrine or any of its chemically related substitutes.

We may conclude that dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) is apparently an effective agent for use in allergic disease. Obvious advantages over other agents ordinarily employed for such conditions are the low toxicity and the freedom from unpleasant cardiovascular and nervous symptoms. Complications due to treatment include somnolence and orthostatic hypotension, either of which may necessitate a decrease in dosage, but these conditions almost never completely preclude the successful use of the drug.

## SUMMARY

1. The effects of dimethylaminoethyl benzhydryl ether hydrochloride have been studied in normal persons, and in those with widely diverse diseases, most of which have been associated with some disturbance of the autonomic nervous system.

2. Results of the following determinations were not abnormally altered in any of the subjects by the administration of the drug: basal metabolism, circulation time, renal function, erythrocyte and leucocyte counts, hemoglobin, differential white counts, hematocrit readings, blood urea nitrogen, creatinine, glucose, proteins, cholesterol, alkaline phosphatase, icteric index, van den Bergh reaction, and cephalin flocculation.

3. An increase in weight occurred in four patients as a result of improvement in their general condition.

4. The majority of subjects showed no change in pulse rate or in blood pressure. Orthostatic hypotension was observed in six subjects. A slight elevation in pulse rate was seen in five patients taking 400 mg. of dimethylaminoethyl benzhydryl ether hydrochloride daily for relatively long periods of time.

5. Pupillary dilatation, accelerated in its appearance but not increased in its amplitude by epinephrine, was observed when the drug was instilled into the conjunctivae.

6. Vital capacity and respiratory rate were uninfluenced by the drug, unless initially lowered as a result of asthma.

7. Capillary permeability was moderately decreased by prolonged treatment with large doses of the alkamine ether compound.

8. Three of ten subjects receiving the drug intravenously developed mild transient reactions.

9. Salivary secretion was apparently not influenced by the range of dosage employed throughout these studies. Both free and total gastric acidity were strikingly decreased. The mouth-anus time was not altered in the normal subjects. Abdominal pain was relieved in ten of seventeen patients.

10. The degree of reduction in the size of the histamine skin reactions was roughly proportional to the daily dose of drug administered. For a brief period after the drug was discontinued, the response to histamine was increased.

11. Definite therapeutic effect was obtained from the drug in certain gastrointestinal neuroses, asthma, functional dysmenorrhea, and dermal allergy.

12. Untoward reactions to benadryl were of mild degree and of infrequent occurrence. They included dizziness, blurring of vision, weakness, and somnolence. Each disappeared promptly upon discontinuing the drug or decreasing the dose.

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# APPLICATION OF THE Rh BLOOD TYPES AND Hr FACTOR IN DISPUTED PARENTAGE

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NOW that the theory of multiple allelic genes[1] for the heredity of the Rh blood types is firmly established,[2, 3] these tests are being applied as a routine alongside of the ordinary blood grouping, subgrouping, and M-N tests in cases of disputed parentage. Sufficient evidence has also accumulated to justify the routine use of the standard Hr factor (Hr′),[4, 5] although sera corresponding to the factor Hr″ recently described in England[6] are not yet generally available. When discussing the application of the Rh blood types and standard Hr factor in the past, our practice has been merely to give the rules of inheritance, from which the possibilities for any particular mating could be derived. The proper application of these laws requires considerable genetic and serologic knowledge, however, and lawyers, in particular, have requested detailed tables listing all the possible matings for reference use. The tables are far more elaborate than in the case of the blood groups and M-N types, and some of the matings involve rare types which will hardly ever be encountered in practical work. Nevertheless, in view of the need for such a complete set of tables, this paper was prepared.

## THE EIGHT Rh BLOOD TYPES

As shown in Table I, with the aid of the three varieties of Rh antisera, anti-$Rh_o$, anti-Rh′ and anti-Rh″, eight types of blood can be distinguished. The factor $Rh_o$ was so designated because it has a special position in relation to the other two Rh factors. Thus, if the reactions of the anti-$Rh_o$ serum are disregarded, a scheme of four types results which is analogous serologically and genetically (except for the rare $Rh_z$ factor) to the four Landsteiner blood groups.

When selecting the designations of the eight Rh blood types, I was guided by the principle of maximum simplicity without ambiguity.[7-10] Thus, blood not agglutinable by any of the three Rh antisera is said to be Rh negative, or, more simply, is said to belong to type rh. Types Rh′, Rh″, Rh′Rh″, and $rh_o$ are named after the antisera with which these bloods react. Type $Rh_1$ reacts with the two antisera, anti-Rh′ and anti-$Rh_o$, and $Rh_1$ is merely an abbreviation for $Rh_o$′; similarly, $Rh_2$ is short for $Rh_o$″. Types $Rh_1$ and $Rh_2$ are so named, instead of $Rh_oRh'$ and $Rh_oRh''$, respectively, to indicate that the two factors $Rh_o$ and Rh′ in the former, and the factors $Rh_o$ and Rh″ in the latter behave like "partial" antigens in complex agglutinogens which are, as a rule, hereditarily transmitted as units by the corresponding genes, *R′*

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TABLE I. CLASSIFICATION OF EIGHT Rh BLOOD TYPES

| BLOOD CONTAINING FACTOR $Rh_o$ | | | | BLOOD NOT CONTAINING FACTOR $Rh_o$ | | | |
|---|---|---|---|---|---|---|---|
| DESIGNATION OF TYPES* | REACTIONS WITH SERA | | | DESIGNATION OF TYPES* | REACTIONS WITH SERA | | |
| | ANTI-Rh′ | ANTI-Rh″ | ANTI-$Rh_o$ | | ANTI-Rh′ | ANTI-Rh″ | ANTI-$Rh_o$ |
| $rh_o$ | − | − | + | rh | − | − | − |
| $Rh_1$ | + | − | + | Rh′ | + | − | − |
| $Rh_2$ | − | + | + | Rh″ | − | + | − |
| $Rh_1Rh_2$ | + | + | + | Rh′Rh″ | + | + | − |

*Type rh was formerly designated Rh negative; type $Rh_1$ contains the two factors $Rh_o$ and Rh′, the name $Rh_1$ being short for $Rh_o$′; similarly, $Rh_2$ is short for $Rh_o$″, and $Rh_1Rh_2$ is short for $Rh_o$′$Rh_o$″.

and $R^z$. Type $rh_o$ is printed with a small "r" to indicate that this type bears a similar relation to types $Rh_1$, $Rh_2$, and $Rh_2Rh_1$ to that which type rh bears to Rh′, Rh″, and Rh′Rh″. In giving the names of the Rh types verbally, however, it is not necessary to say small "r" or large "r," as the case may be, because the qualifying subscripts and superscripts will prevent any ambiguity.

According to the theory of six allelic genes,[1] the eight Rh blood types are hereditarily transmitted by means of a series of allelic genes, $r$, $R'$, $R''$, $r^0$, $R^1$, and $R^z$. It will be noticed that in order to avoid confusion between the names of the Rh genes and genotypes on the one hand, and the Rh agglutinogens and phenotypes on the other, the letter "h" is omitted, and italics are used in the designations of the former. The uniform use of qualifying superscripts in the gene symbols is necessary in order to conform with the practice of geneticists when naming genes belonging to a single allelic series. Under the six-gene theory, twenty-one genotypes are possible, and the way in which these correspond with the eight standard Rh types is shown in Table II.

TABLE II. EIGHT Rh TYPES AND THEIR TWENTY-ONE GENOTYPES*

| Rh BLOOD TYPES | POSSIBLE GENOTYPES |
|---|---|
| rh | $rr$ |
| Rh′ | $R'R'$ and $R'r$ |
| Rh″ | $R''R''$ and $R''r$ |
| Rh′Rh″ | $R'R''$ |
| $rh_o$ | $r^0r^0$ and $r^0r$ |
| $Rh_1$ | $R^1R^1$, $R^1R'$, $R^1r$, $R^1r^0$, and $R'r^0$ |
| $Rh_2$ | $R^zR^z$, $R^zR''$, $R^zr$, $R^zr^0$, and $R''r^0$ |
| $Rh_1Rh_2$ | $R^1R^z$, $R^1R''$, and $R'R^z$ |

*Does not take into account the rare $R^z$ gene or the rare intermediate genes.

When the Rh blood types are applied in cases of disputed parentage, the practical consequences of the theory of six allelic genes can be summarized in the following two rules: (1) The factors $Rh_o$, Rh′, and Rh″ cannot appear in the blood of a child unless present in the blood of one or both parents. (2) When either parent belongs to type $Rh_1Rh_2$ or Rh′Rh″, no child of type rh or $rh_o$ can occur. Similarly, parents of type rh or $rh_o$ cannot have children of type $Rh_1Rh_2$ or Rh′Rh″.

In studies of the eight Rh blood types on a total of 300 families with 620 children, we have not encountered a single exception to these two rules of heredity.[2, 3, 10] The theory of six allelic genes is also supported by statistical studies on the distribution of the Rh blood types in the general population.[11]

TABLE III. EIGHT Rh BLOOD TYPES IN DISPUTED PARENTAGE

| MATING | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE |
|---|---|---|---|
| rh × rh | rh | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh′, Rh″, and Rh′Rh″ | |
| rh × Rh′ | rh and Rh′ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″ | |
| rh × Rh″ | rh and Rh″ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh′, and Rh′Rh″ | |
| rh × Rh′Rh″ | Rh′ and Rh″ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$ | Rh′Rh″ and rh |
| rh × $rh_o$ | rh and $rh_o$ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, Rh′, Rh″, and Rh′Rh″ | |
| rh × $Rh_1$ | $Rh_1$, Rh′, $rh_o$, and rh | $Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″ | |
| rh × $Rh_2$ | $Rh_2$, Rh″, $rh_o$, and rh | $Rh_1$, Rh′, $Rh_1Rh_2$, and Rh′Rh″ | |
| rh × $Rh_1Rh_2$ | $Rh_1$, $Rh_2$, Rh′, and Rh″ | | $Rh_1Rh_2$, Rh′Rh″, $rh_o$, and rh |
| Rh′ × Rh′ | rh and Rh′ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″ | |
| Rh′ × Rh″ | Rh′, Rh″, Rh′Rh″, and rh | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$ | |
| Rh′ × Rh′Rh″ | Rh′, Rh″, and Rh′Rh″ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$ | rh |
| Rh′ × $rh_o$ | $Rh_1$, Rh′, $rh_o$, and rh | $Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″ | |
| Rh′ × $Rh_1$ | $Rh_1$, Rh′, $rh_o$, and rh | $Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″ | |
| Rh′ × $Rh_2$ | $Rh_1$, $Rh_2$, Rh′, Rh″, $Rh_1Rh_2$, Rh′Rh″, $rh_o$, and rh | | |
| Rh′ × $Rh_1Rh_2$ | $Rh_1$, $Rh_2$, Rh′, Rh″, $Rh_1Rh_2$, and Rh′Rh″ | | $rh_o$ and rh |
| Rh″ × Rh″ | Rh″ and rh | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh′, and Rh′Rh″ | |
| Rh″ × Rh′Rh″ | Rh′, Rh″, and Rh′Rh″ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$ | rh |
| Rh″ × $rh_o$ | $Rh_2$, Rh″, $rh_o$, and rh | $Rh_1$, Rh′, $Rh_1Rh_2$, and Rh′Rh″ | |
| Rh″ × $Rh_1$ | $Rh_1$, $Rh_2$, Rh′, Rh″, $Rh_1Rh_2$, Rh′Rh″, $rh_o$, and rh | | |
| Rh″ × $Rh_2$ | $Rh_2$, Rh″, $rh_o$, and rh | $Rh_1$, Rh′, $Rh_1Rh_2$, and Rh′Rh″ | |
| Rh″ × $Rh_1Rh_2$ | $Rh_1$, $Rh_2$, Rh′, Rh″, $Rh_1Rh_2$, and Rh′Rh″ | | $rh_o$ and rh |

*(Continued on next page.)*

TABLE III—CONT'D

| MATING | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE |
|---|---|---|---|
| $Rh'Rh'' \times Rh'Rh''$ | $Rh'$, $Rh''$, $Rh'Rh''$ | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$ | rh |
| $Rh'Rh'' \times rh_o$ | $Rh_1$, $Rh'$, $Rh_2$, and $Rh''$ | | $Rh_1Rh_2$, $Rh'Rh''$, $rh_o$ and rh |
| $Rh'Rh'' \times Rh_1$ | $Rh_1$, $Rh'$, $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $rh_o$ and rh |
| $Rh'Rh'' \times Rh_2$ | $Rh_1$, $Rh'$, $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $rh_o$ and rh |
| $Rh'Rh'' \times Rh_1Rh_2$ | $Rh_1$, $Rh'$, $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $rh_o$ and rh |
| $rh_o \times rh_o$ | $rh_o$ and rh | $Rh_1$, $Rh_2$, $Rh_1Rh_2$, $Rh'$, $Rh''$, and $Rh'Rh''$ | |
| $rh_o \times Rh_1$ | $Rh_1$, $Rh'$, $rh_o$, and rh | $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | |
| $rh_o \times Rh_2$ | $Rh_2$, $Rh''$, $rh_o$, and rh | $Rh_1$, $Rh'$, $Rh_1Rh_2$, and $Rh'Rh''$ | |
| $rh_o \times Rh_1Rh_2$ | $Rh_1$, $Rh_2$, $Rh'$, and $Rh''$ | | $Rh_1Rh_2$, $Rh'Rh''$, $rh_o$, and rh |
| $Rh_1 \times Rh_1$ | $Rh_1$, $Rh'$, $rh_o$, and rh | $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | |
| $Rh_1 \times Rh_2$ | $Rh_1$, $Rh_2$, $Rh'$, $Rh''$, $Rh_1Rh_2$, $Rh'Rh''$, $rh_o$, and rh | | |
| $Rh_1 \times Rh_1Rh_2$ | $Rh_1$, $Rh_2$, $Rh'$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $rh_o$ and rh |
| $Rh_2 \times Rh_2$ | $Rh_2$, $Rh''$, $rh_o$, and rh | $Rh_1$, $Rh'$, $Rh_1Rh_2$, and $Rh'Rh''$ | |
| $Rh_2 \times Rh_1Rh_2$ | $Rh_1$, $Rh_2$, $Rh'$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $rh_o$ and rh |
| $Rh_1Rh_2 \times Rh_1Rh_2$ | $Rh_1$, $Rh_2$, $Rh'$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $rh_o$ and rh |

However, that rare but valid exceptions to the second rule of heredity may eventually be encountered follows from the discovery of the rare $R^z$ gene by Murray, Race, and Taylor[12] and of the so-called intermediate genes by me.[13] Accordingly, when applying the Rh tests in disputed parentage, while any contradiction to the first rule of heredity should be considered reliable proof of nonparentage, a contradiction to the second rule may be only considered as evidence that parentage is very improbable.

In Table III, are listed all the possible matings of the eight Rh blood types, giving the types possible among the children under the six-gene theory. In the third column of Table III are given the types which cannot occur in the children based on the first rule; in such instances parentage is definitely excluded. In the last column are given the types excluded by the second rule; in such instances parentage is highly improbable, as has just been explained.

### THE STANDARD Hr FACTOR AND THE EIGHT Rh TYPES

As has already been mentioned, the standard Hr factor (or the factor Hr′) is the only Hr factor generally available at the present time for practical use in clinical and legal medicine. According to the theory of Race and Taylor,[14] and Race and associates,[15] which is now well established, this factor occurs as a partial antigen in the agglutinogens determined by genes $r$, $r^0$, $R'''$, and $R^2$ and is absent from the agglutinogens determined by genes $R'$ and $R^1$ (and $R^y$ and $R^z$). Briefly, therefore, standard Hr is related to the Rh′ factor in the same way that M is related to N, and, to indicate this, the standard Hr factor was designated Hr′ by me.[5]

TABLE IV. CLASSIFICATION OF Rh BLOOD TYPES AND SUBTYPES

| Rh blood types | Reactions with sera: anti-Rh′ | anti-Rh″ | anti-Rh$_o$ | Genotypes | Reactions with sera: anti-Hr′ | anti-Hr″ | Rh subtypes | Approximate distribution (per cent among Caucasians in New York) | |
|---|---|---|---|---|---|---|---|---|---|
| rh | Neg. | Neg. | Neg. | $rr$ | Pos. | Pos. | ---- | 13.0 | |
| Rh′ | Pos. | Neg. | Neg. | $R'R'$ | Neg. | Pos. | Rh′Rh′ | .01 | 1.0 |
| | | | | $R'r$ | Pos. | Pos. | Rh′rh | 1.0 | |
| Rh″ | Neg. | Pos. | Neg. | $R''R''$ | Pos. | Neg. | Rh″Rh″ | .005 | 0.5 |
| | | | | $R''r$ | Pos. | Pos. | Rh″rh | 0.5 | |
| Rh′Rh″ | Pos. | Pos. | Neg. | $R'R''$ | Pos. | Pos. | ---- | .01 | |
| rh$_o$ | Neg. | Neg. | Pos. | $r^0r^0$, $r^0r$ | Pos. | Pos. | ---- | 2.0 | |
| Rh$_1$ (Rh$'_o$) | Pos. | Neg. | Pos. | $R^1R^1$, $R^1R'$ | Neg. | Pos. | Rh$_1$Rh$_1$ | 20.0 | 54.0 |
| | | | | $R^1r$, $R^1R^0$, $R'r^0$ | Pos. | Pos. | Rh$_1$rh | 34.0 | |
| Rh$_2$ (Rh$''_o$) | Neg. | Pos. | Pos. | $R^2R^2$, $R^2R''$ | Pos. | Neg. | Rh$_2$Rh$_2$ | 3.0 | 15.0 |
| | | | | $R^2r$, $R^2r^0$, $R''r^0$ | Pos. | Pos. | Rh$_2$rh | 12.0 | |
| Rh$_1$Rh$_2$ | Pos. | Pos. | Pos. | $R^1R^2$, $R'R^2$, $R^1R''$ | Pos. | Pos. | ---- | 14.5 | |

As shown in Table IV, with the aid of anti-Hr′ serum, the types Rh$_1$ and Rh′ can each be divided into two subtypes, thus increasing the number of identifiable types of blood from eight to ten. In a similar manner, with the aid of anti-Hr″ serum, it will be possible to subdivide types Rh$_2$ and Rh″, further increasing the total number of Rh types to twelve. The designations of the subtypes were selected to indicate the most common genotype occurring among individuals belonging to the subtype in question. Again the designations have been made as simple as possible, and there is still no danger of ambiguity, because, for example, the designations of phenotypes Rh$_1$Rh$_1$ and Rh$_1$rh could hardly be confused with the designations of genotypes $R^1R^1$ and $R^1r$.

TABLE V. EXCLUSIONS OF PARENTAGE POSSIBLE WITH AID OF STANDARD ANTI-Hr SERA*

| MATING | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE |
|---|---|---|---|
| rh × Rh′Rh′ | Rh′rh | rh, Rh′Rh′ ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″) | |
| rh × Rh′rh | Rh′rh and rh | Rh′Rh′ ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″) | |
| rh × Rh′Rh″ | Rh′rh and Rh″ | Rh′Rh′ ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | (Rh′Rh″ and rh) |
| rh × $Rh_1Rh_1$ | $Rh_1$rh and Rh′rh | $Rh_1Rh_1$, Rh′Rh′, $rh_o$, rh ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| rh × $Rh_1$rh | $Rh_1$rh, Rh′rh, $rh_o$, and rh | $Rh_1Rh_1$, Rh′Rh′ ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| rh × $Rh_1Rh_2$ | $Rh_1$rh, Rh′rh, $Rh_2$, and Rh″ | $Rh_1Rh_1$, Rh′Rh′ | (Rh$_1$Rh$_2$, Rh′Rh″, $rh_o$, and rh) |
| Rh′Rh′ × Rh′Rh′ | Rh′Rh′ | Rh′rh, rh ($Rh_1$, $Rh_2$ $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″) | |
| Rh′Rh′ × Rh′rh | Rh′Rh′ and Rh′rh | rh ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″) | |
| Rh′rh × Rh′rh | Rh′Rh′, Rh′rh, and rh | ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, $rh_o$, Rh″, and Rh′Rh″) | |
| Rh′Rh′ × Rh″ | Rh′rh and Rh′Rh″ | Rh′Rh′, Rh″, rh ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | |
| Rh′rh × Rh″ | Rh′rh, Rh′Rh″, Rh″, and rh | Rh′Rh′ ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | |
| Rh′Rh′ × Rh′Rh″ | Rh′Rh′ and Rh′Rh″ | Rh′rh, Rh″ ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | (rh) |
| Rh′rh × Rh′Rh″ | Rh′Rh′, Rh′rh, Rh″, and Rh′Rh″ | ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | (rh) |
| Rh′Rh′ × $rh_o$ | $Rh_1$rh and Rh′rh | $Rh_1Rh_1$, Rh′Rh′, $rh_o$, rh ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| Rh′rh × $rh_o$ | $Rh_1$rh, Rh′rh, $rh_o$, and rh | $Rh_1Rh_1$, Rh′Rh′ ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| Rh′Rh′ × $Rh_1Rh_1$ | $Rh_1Rh_1$, and Rh′Rh′ | $Rh_1$rh, Rh′rh, $rh_o$, rh ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| Rh′Rh′ × $Rh_1$rh | $Rh_1Rh_1$, $Rh_1$rh, Rh′Rh′, and Rh′rh | $rh_o$, rh ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| Rh′rh × $Rh_1Rh_1$ | $Rh_1Rh_1$, $Rh_1$rh, Rh′Rh″, and Rh′rh | $rh_o$, rh ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| Rh′rh × $Rh_1$rh | $Rh_1Rh_1$, $Rh_1$rh, Rh′Rh′, Rh′rh, $rh_o$, and rh | ($Rh_2$, Rh″, $Rh_1Rh_2$, and Rh′Rh″) | |
| Rh′Rh′ × $Rh_2$ | $Rh_1$rh, Rh′rh, $Rh_1Rh_2$, and Rh′Rh″ | $Rh_2$, Rh″, $rh_o$, rh, $Rh_1Rh_1$, and Rh′Rh′ | |
| Rh′rh × $Rh_2$ | $Rh_1$rh, Rh′rh, $Rh_1Rh_2$, Rh′Rh″, $Rh_2$, Rh″, $rh_o$, and rh | $Rh_1Rh_1$, and Rh′Rh′ | |
| Rh′Rh′ × $Rh_1Rh_2$ | $Rh_1Rh_1$, Rh′Rh′, $Rh_1Rh_2$, and Rh′Rh″ | $Rh_2$, Rh″, $rh_o$ and rh | $Rh_1$rh and Rh′rh |

*Types enclosed in parentheses are those excluded under the theory of six standard genes, without the aid of anti-Hr serum (see Table III).

TABLE V—CONT'D

| MATING | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED | IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE |
|---|---|---|---|
| $Rh'rh \times Rh_1Rh_2$ | $Rh_2Rh_1$, $Rh_1rh$, $Rh'Rh'$, $Rh'rh$, $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | ($rh_o$ and rh) |
| $Rh'' \times Rh'Rh''$ | $Rh'rh$, $Rh''$, and $Rh'Rh''$ | $Rh'Rh'$ ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | (rh) |
| $Rh'' \times Rh_1Rh_1$ | $Rh_1Rh_2$, $Rh_1rh$, $Rh'Rh''$, and $Rh'rh$ | $Rh_1Rh_1$, $Rh'Rh'$, $Rh_2$, $Rh''$, $rh_o$, and rh | |
| $Rh'' \times Rh_1rh$ | $Rh_1Rh_2$, $Rh_1rh$, $Rh'Rh''$, $Rh'rh$, $Rh_2$, $Rh''$, $rh_o$, and rh | $Rh_1Rh_1$, and $Rh'Rh'$ | |
| $Rh'' \times Rh_1Rh_2$ | $Rh_1Rh_2$, $Rh'Rh''$, $Rh_1rh$, $Rh'rh$, $Rh_2$, and $Rh''$ | $Rh_1Rh_1$, and $Rh'Rh'$ | ($rh_o$ and rh) |
| $Rh'Rh'' \times Rh'Rh''$ | $Rh'Rh'$, $Rh''$, and $Rh'Rh''$ | ($Rh_1$, $Rh_2$, $Rh_1Rh_2$, and $rh_o$) | $Rh'rh$ (rh) |
| $Rh'Rh'' \times rh_o$ | $Rh_1rh$, $Rh'rh$, $Rh_2$, and $Rh''$ | $Rh_1Rh_1$ and $Rh'Rh'$ | ($Rh_1Rh_2$, $Rh'Rh''$, $rh_o$, and rh) |
| $Rh'Rh'' \times Rh_1Rh_1$ | $Rh_1Rh_1$, $Rh'Rh'$, $Rh_1Rh_2$, and $Rh'Rh''$ | $Rh_2$, $Rh''$, $rh_o$, and rh | $Rh_1rh$, $Rh'rh$ |
| $Rh'Rh'' \times Rh_1rh$ | $Rh_1Rh_1$, $Rh_1rh$, $Rh'Rh'$, $Rh'rh$, $Rh_1Rh_2$, $Rh'Rh''$, $Rh_2$, and $Rh''$ | | ($rh_o$ and rh) |
| $Rh'Rh'' \times Rh_1Rh_2$ | $Rh_1Rh_1$, $Rh'Rh'$, $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | | $Rh_1rh$, $Rh'rh$ ($rh_o$ and rh) |
| $rh_o \times Rh_1Rh_1$ | $Rh_1rh$ and $Rh'rh$ | $Rh_1Rh_1$, $Rh'Rh'$, $rh_o$, rh ($Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$) | |
| $rh_o \times Rh_1rh$ | $Rh_1rh$, $Rh'rh$, $rh_o$, and rh | $Rh_1Rh_1$, $Rh'Rh'$ ($Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$) | |
| $rh_o \times Rh_1Rh_2$ | $Rh_1rh$, $Rh'rh$, $Rh_2$, and $Rh''$ | $Rh_1Rh_1$ and $Rh'Rh'$ | ($Rh_1Rh_2$, $Rh'Rh''$, $rh_o$, and rh) |
| $Rh_1Rh_1 \times Rh_1Rh_1$ | $Rh_1Rh_1$ and $Rh'Rh'$ | $Rh_1rh$, $Rh'rh$, $rh_o$, rh ($Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$) | |
| $Rh_1Rh_1 \times Rh_1rh$ | $Rh_1Rh_1$, $Rh_1rh$, $Rh'Rh'$, and $Rh'rh$ | $rh_o$, rh ($Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$) | |
| $Rh_1rh \times Rh_1rh$ | $Rh_1Rh_1$, $Rh_1rh$, $Rh'Rh'$, $Rh'rh$, $rh_o$, and rh | ($Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$) | |
| $Rh_1Rh_1 \times Rh_2$ | $Rh_1rh$, $Rh_1Rh_2$, $Rh'rh$, and $Rh'Rh''$ | $Rh_1Rh_1$, $Rh'Rh'$, $Rh_2$, $Rh''$, $rh_o$, and rh | |
| $Rh_1rh \times Rh_2$ | $Rh_1rh$, $Rh_1Rh_2$, $Rh'rh$, $Rh'Rh''$, $Rh_2$, $Rh''$, $rh_o$, and rh | $Rh_1Rh_1$ and $Rh'Rh'$ | |
| $Rh_1Rh_1 \times Rh_1Rh_2$ | $Rh_1Rh_1$, $Rh'Rh'$, $Rh_1Rh_2$, and $Rh'Rh''$ | $Rh_2$, $Rh''$, $rh_o$, and rh | $Rh_1rh$ and $Rh'rh$ |
| $Rh_1rh \times Rh_1Rh_2$ | $Rh_1Rh_1$, $Rh_1rh$, $Rh'Rh'$, $Rh'rh$, $Rh_1Rh_2$, $Rh'Rh''$, $Rh_2$ and $Rh''$ | | ($rh_o$ and rh) |
| $Rh_2 \times Rh_1Rh_2$ | $Rh_1rh$, $Rh'rh$, $Rh_2$, $Rh''$, $Rh_1Rh_2$, and $Rh'Rh''$ | $Rh_1Rh_1$ and $Rh'Rh'$ | ($rh_o$ and rh) |
| $Rh_1Rh_2 \times Rh_1Rh_2$ | $Rh_1Rh_1$, $Rh'Rh'$, $Rh_1Rh_2$, $Rh'Rh''$, $Rh_2$, and $Rh''$ | | $Rh_1rh$, $Rh'rh$ ($rh_o$ and rh) |

The theory of Race and Taylor leads to the following two additional rules of heredity, which are of practical value in problems of disputed parentage: (1) The factor Hr′ cannot appear in the blood of a child unless present in the blood of one or both parents. (2) Parents of type $Rh_1Rh_1$ or Rh′Rh′ cannot have children of type rh, $rh_0$, Rh″, or $Rh_2$. Similarly, parents of type rh, $rh_0$, Rh″, or $Rh_2$ cannot have children of type $Rh_1Rh_1$ or Rh′Rh′.

In a study of the Hr factor and Rh blood types in a series of eighty-one families with 127 children, Sonn, Polivka, and I have not encountered a single contradiction of these two laws of heredity.[10] Similar results have been obtained by Race and associates[16, 17] in studies on a larger series of families. Moreover, investigations on the distribution of the Hr factor and Rh blood types in the general population have also yielded results in conformity with the theoretical expectations.[4, 5] Therefore, it seems justified to apply these two laws without qualification in cases of disputed parentage. In fact, an exclusion of parentage based on the Rh-Hr tests has already been accepted by at least one court as the principal basis for the acquittal of a falsely accused man in a paternity proceeding.

The practical conclusions that can be drawn from the two laws of heredity when applying the Hr-Rh tests in cases of disputed parentage are presented in Table V. By increasing the number of identifiable Rh blood types from eight to ten, the use of anti-Hr serum has considerably increased the number of possible matings which must be included in such a table. We have omitted from Table V those matings, such as rh × rh and rh × Rh″, which are given in Table III and in which the use of standard anti-Hr serum does not affect the possibilities. In the remaining matings listed in Table V, those types which are excluded among the children on the basis of the six-gene theory without the aid of anti-Hr serum have been placed in parentheses, in order to separate them from the types excluded on the basis of the Rh-Hr theory just discussed.

## SUMMARY

Tables I to V have been presented in order to facilitate the application of the Rh blood types and standard Hr factor in cases of disputed parentage.

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# THE RELATION OF PHYSIOLOGIC VARIABLES TO CIRCULATION TIME

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CIRCULATION times reported in the literature on presumably normal subjects exhibit very wide variations. Bernstein and Simkins,[1] using magnesium sulfate, found a range of from 7 to 18 seconds. Kvale and Allen,[2] using a "calcium and magnesium mixture," found a range of from 5 to 24 seconds, with an average of 13.7 seconds. Smith, Allen, and Craig,[3] using sodium cyanide, reported a range of from 12.4 to 33 seconds, with an average of 20 seconds. Such wide ranges in normal values make it obvious that one could attach significance to a measurement of circulation time only when the value obtained differs very widely from the average. The measurements reported here were made in an attempt to find some other easily measured factor or factors having a definite correlation with the circulation time, with the idea that such a finding would make it possible to narrow the range of circulation time for any specific subject and thus give added significance to the test.

## METHODS

All measurements were done under basal conditions on fifty healthy medical students who understood the nature of the tests and cooperated freely. In addition to circulation time, determinations were made on (1) basal metabolic rate, (2) pulse rate, (3) respiratory rate, (4) tidal air volume, (5) vital capacity, (6) arterial blood pressure, (7) pulse pressure, (8) breath-holding time, (9) minute respiratory volume, and (10) body surface area.

The circulation time was determined by using aminophylline‡ as the reagent, as was suggested by Koster and Sarnoff,[4] with the exception that 0.5 ml. containing 120 mg. of aminophylline was used rather than 1.0 ml. containing 240 mg. as they suggested. The time recorded as circulation time was from the beginning of the injection to the reaction. The reaction, or end point, is purely objective and can easily be taken before the subjective reaction occurs. The end point is usually a sharp inspiration (prolonged and deep) which may be initiated at any phase of the respiratory cycle. When grimaces or swallows preceded the inspirations, they were used as end points. Such reactions were uncommon.

The basal metabolic rate was determined by using the Benedict-Roth apparatus, the only deviation from the accepted methods being that the students

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were not confined to the building for the night preceding the test morning but were allowed to present themselves at the laboratory after having carried out the other accepted precautions.

The respiratory minute volume was calculated from the tracing obtained in measuring the basal metabolic rate, using an interval of from 3 to 6 minutes, depending upon the regularity of the tracing.

The breath-holding time was measured by giving the subject a stop watch and instructions to determine how long he could hold his breath. There was no period of previous hyperventilation, and the subject was entirely alone and therefore not on a competitive basis. The test was usually terminated when the subject experienced a moderate discomfort.

The other measurements were carried out by accepted standard procedures.

In order to standardize the results as much as possible, each of us performed the same specific duty for each measurement.

## RESULTS

The results obtained are summarized in Table I which shows the range, the mean, and the correlation coefficient of each of the tests with circulation time.

Examination of Table I shows that the correlation coefficient between the pulse rate and circulation time is −0.52, which indicates an inverse relation between these values. This value of the correlation coefficient is well above the 1 per cent level and is therefore significant.[5] The correlation coefficient between breath-holding time and circulation time is +0.33, which indicates a probably significant direct correlation.

All of the other measurements, including that of the body surface area in square meters, the correlation coefficient of which is not given, failed to show any significant correlation with circulation time.

TABLE I. VALUES FOR PHYSIOLOGIC VARIABLES IN FIFTY NORMAL YOUNG ADULTS AND CORRELATION COEFFICIENTS FOR EACH WITH CIRCULATION TIME

| | LOW | HIGH | MEAN | CORRELATION COEFFICIENT |
|---|---|---|---|---|
| Circulation time | 5.5 | 32.8 | 16.0 | |
| Reclining pulse rate | 44.0 | 100.0 | 71.0 | −0.52 |
| Basal metabolic rate | 70.0 | 122.0 | 92.6 | −0.2061 |
| Room temperature (° C.) | 21.0 | 29.0 | 24.7 | −0.184 |
| Oral temperature (° F.) | 97.6 | 100.0 | 98.57 | −0.144 |
| Minute volume (Liters) | 3.25 | 11.81 | 5.46 | −0.100 |
| Vital capacity (Milliliters) | 2678.0 | 6556.0 | 4283.0 | +0.059 |
| Breath-holding time (Seconds) | 33.0 | 167.0 | 69.0 | +0.333 |
| Reclining respiratory rate | 8.0 | 20.0 | 14.0 | +0.0019 |
| Standing respiratory rate | 8.0 | 22.0 | 16.0 | −0.0064 |
| Reclining diastolic blood pressure | 56.0 | 90.0 | 76.6 | −0.2601 |
| Reclining systolic blood pressure | 98.0 | 140.0 | 117.4 | −0.1880 |
| Reclining pulse pressure | 20.0 | 64.0 | 40.0 | −0.0165 |
| Standing diastolic blood pressure | 72.0 | 100.0 | 87.9 | −0.1950 |
| Standing systolic blood pressure | 104.0 | 148.0 | 121.4 | −0.2670 |
| Standing pulse pressure | 16.0 | 64.0 | 33.3 | −0.1060 |
| Distance from cubital fossa to sternal notch (Cm.) | 36.0 | 50.0 | 43.7 | +0.0136 |

## DISCUSSION

As is indicated in Table I, we observed in young healthy adults a circulation time which averaged 16 seconds with a range of from 5.5 to 32.8 seconds. Using the same reagent, Koster and Sarnoff[4] observed a range of from 7.1 to 20.4, with a mean of 12.4 seconds. They used as subjects postoperative patients with no cardiovascular disease. Bellis, Doss, and Craft[6] observed a decreased circulation time following operation. This they attributed to increased metabolic demands of the organism, which is manifested by increased temperature, pulse rate, and respiratory rate. Smith and Allen[7] also reported that circulation time is prolonged after the fourth postoperative day but is usually decreased during the first twenty-four to forty-eight hours. Feinsilver[8] found the circulation time decreased following chest operations when the patient was given sufficient time to adjust to the new condition. The observations of these various workers concerning postoperative patients may account for the difference of our mean as compared with that of Koster and Sarnoff.[4]

The following observations with regard to pulse rate and its relation to circulation time, though not treated statistically by the various workers, seem to substantiate our results. Averbuck and Friedman[9] attribute the decreased circulation time in children to the rapid heart rate and/or the short distance, but the latter in our experience shows no correlation. Macy, Claiborne, and Hurxthal,[10] using decholin in normal subjects, observed an average circulation time of 13.0 seconds, but the pulse rate averaged 87, which is 16.0 beats per minute higher than we observed, with an average circulation time of 16.0 seconds. Kvale and Allen[11] observed that atropine-induced tachycardia in human subjects, with little change in blood pressure or skin temperature, produced a decrease in circulation time.

To our knowledge, no attempt at correlation of circulation time to breath-holding time has been reported previously. This correlation in itself may be of no significance but is mentioned as an interesting observation. Though there seems to be a significant direct relationship between circulation time and breath-holding time, this is applicable only in the normal subject, as it is a well-known fact that patients with dyspnea of cardiac origin are obviously short of breath but have prolonged circulation times.

Attention is called to the average basal metabolic rate of 92.6 per cent. Although this value is definitely lower than the usually accepted mean, such a figure has been repeatedly shown to be compatible with this geographic area. Inasmuch as it would seem logical to assume that the metabolic demands would be reflected in the cardiac output and hence in the circulation time, we were somewhat surprised to find that basal metabolic rate showed no correlation with circulation time. However, a review of the literature fully supports our findings. Macy, Claiborne, and Hurxthal[10] observed that circulation time was increased in hypothyroidism but not in hypometabolic states without myxedema and not in hypopituitarism. Baer and Isard[12] found that circulation time could be correlated with basal metabolic rate in hypothyroid and hyperthyroid states.

but they found no such correlation in other glandular diseases, in postmenopausal hypertension, or in normal subjects. Although they did not conclude this, these facts are noted if their graphs are carefully studied. Goldberg,[13] Kvale, and Allen[2] have shown that there is almost an absolute inverse relation of circulation time to basal metabolic rate in hyperthyroidism. There seems to be, therefore, some specific effect of the thyroid states on circulation time as compared with other metabolic disturbances and with the normal. This is further borne out by Boothby and Rynearson[14] who noted that the cardiac output is greater in hyperthyroidism with a given oxygen consumption than in a normal subject exercised to bring his oxygen consumption to the same level.

The other physiologic variables listed in Table I show no significant correlation with circulation time.

## SUMMARY AND CONCLUSIONS

Circulation time with the use of aminophylline was determined in fifty healthy young adults. The mean time was 16 seconds, with a range of from 5.5 to 32.8 seconds.

At the same time on the same subjects, the following measurements were made: reclining pulse rate, basal metabolic rate, oral temperature, minute respiratory volume, vital capacity, breath-holding time, respiratory rate, reclining and standing systolic, diastolic, and pulse pressures, and distance from cubital fossa to sternal notch.

Correlation coefficients between each of these measurements and circulation time were determined, revealing a significant inverse correlation between pulse rate and circulation time and a probably significant direct correlation between circulation time and breath-holding time. No other correlations were significant.

No untoward reactions to aminophylline in the dosage used were encountered.

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# *LABORATORY METHODS*

## A PRIMARY STANDARD FOR THE COLORIMETRIC DETERMINATION OF HEMOGLOBIN

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THE methods in general clinical use for the determination of hemoglobin fall into two categories: the older visual methods in which the color of the blood preparation obtained is compared with a colored standard and the photoelectric methods in which the light absorption of the solution is measured by means of a colorimeter which has previously been calibrated, usually by the manufacturer of the instrument. Neither of these methods has proved wholly adequate, since the colored standards may not accurately represent the color given by the blood solutions and are subject to changes as a result of aging, while the photoelectric instruments cannot be relied upon to retain their original calibration over an indefinite period of time. The previously available methods for the standardization of hemoglobin solutions, such as the determination of iron content or gas capacity, are too cumbersome for the average clinical laboratory.

Several methods based on the use of crystalline hemin as the primary standard have recently been suggested for the standardization of hemoglobin. Clegg and King[1] and King, Gilchrist, and Delory[2] have used solutions of crystalline hemin in N/10 NaOH as a primary standard for the determination of hemoglobin as alkaline hematin. Drabkin[3] recommends hemin dicyanide prepared from hemin as a primary standard for the determination of hemoglobin as cyanmethemoglobin. While this latter method is probably the most precise of the colorimetric procedures, there is some objection to the routine use of cyanide, particularly in hospital laboratories.

In this laboratory we have employed the alkaline hematin method, with standards prepared by solution of crystalline hemin in N/10 borate buffer at pH 9.4. These standard solutions are superior to the more alkaline solutions used by King and his co-workers in that they are more stable and have absorption spectra more closely resembling the spectrum of alkaline hematin prepared from whole blood. Such solutions have a further advantage in that they will not react appreciably with glass; they can therefore be kept in sealed tubes which might be used directly in the colorimeter for checking the calibration values. The stability of dilute crystalline hemin solutions sealed into colorimeter tubes is now under investigation, and the results will be described in a later communication.

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## EXPERIMENTAL

*Hematin Solutions.*—Alkaline hematin was prepared from human blood according to the method of Clegg and King, using oxalated blood samples standardized by means of the Beckman spectrophotometer. The blood samples were diluted 1:251 in N/10 NaOH, heated for five minutes in a boiling water bath, and immediately cooled. It has been established that under these conditions the conversion of hemoglobin to alkaline hematin is complete and reproducible.[1]

Crystalline hemin was prepared from sheep blood according to the procedure of Clifcorn, Melocke, and Elvehjem,[4] and a commercial preparation was obtained.* The iron content of these preparations was determined by a microgravimetric method and by the colorimetric method described by Delory.[5] The results are given in Table I. Both preparations gave identical results; those reported in the following sections were obtained with the commercial preparation.

TABLE I

| | Fe CONTENT | PURITY |
|---|---|---|
| Theoretical | 8.57 per cent | |
| Sheep hemin, gravimetric | 8.64 per cent | 101 per cent |
| Sheep hemin, colorimetric | 8.57 per cent | 100 per cent |
| Commercial hemin, gravimetric | 8.55 per cent | 100 per cent |
| Commercial hemin, colorimetric | 8.37 per cent | 98 per cent |

Stock solutions of crystalline hemin containing about 1 mg. per cubic centimeter were prepared by dissolving accurately weighed quantities in N/10 borate buffer at pH 9.4[6] These solutions were allowed to age in the refrigerator and then were accurately diluted with the borate buffer to give solutions of final concentration ranging from 10 to 50 mg. per liter. The spectrophotometric and colorimetric measurements were made on the same day the dilutions were prepared.

For the spectrophotometric measurements, nine solutions diluted from four stock hemin solutions were used; for the colorimetric measurements, a total of fourteen solutions diluted from four stock solutions were prepared. The stock solutions had been aged from one to thirty days. Twelve standardized blood samples, diluted in duplicate with 1:251 blood diluting pipettes, were used in the calibration of the colorimeters.

*Absorption Spectra.*—The absorption spectra were determined with a Beckman spectrophotometer using 10 mm. Corex cells and an effective slit width of 40 A. In Fig. 1 are compared the absorption spectra of the various preparations of alkaline hematin. It will be observed that alkaline hematin prepared by dissolving crystalline hemin in N/10 NaOH (Curve II) differs considerably from alkaline hematin prepared from whole blood (Curve I), particularly below 5,000 A and between 5,500 and 6,000 A. These differences are largely absent in the case of solutions in N/10 borate buffer at pH 9.4 (Curves III and IV).

*Stability of the Crystalline Hemin Solutions.*—As has been pointed out by Collier,[7] solutions of crystalline hemin in N/10 NaOH undergo a progressive change resulting in increases in absorption in the blue and red. The spectrum

*From the Eastman Kodak Company, Rochester, N. Y.

gradually approaches that of hemin dissolved at pH 9.4, although the solutions do not become stable even after two months at room temperature. In the case of solutions buffered at pH 9.4, a small initial change is observed during the first several hours (compare Curves III and IV), but the solutions then appear to be stable. Since this preliminary change proceeds very slowly in dilute solution, stock solutions containing 1 mg. per cubic centimeter of hemin are kept overnight before they are diluted for the absorption measurement. These stock

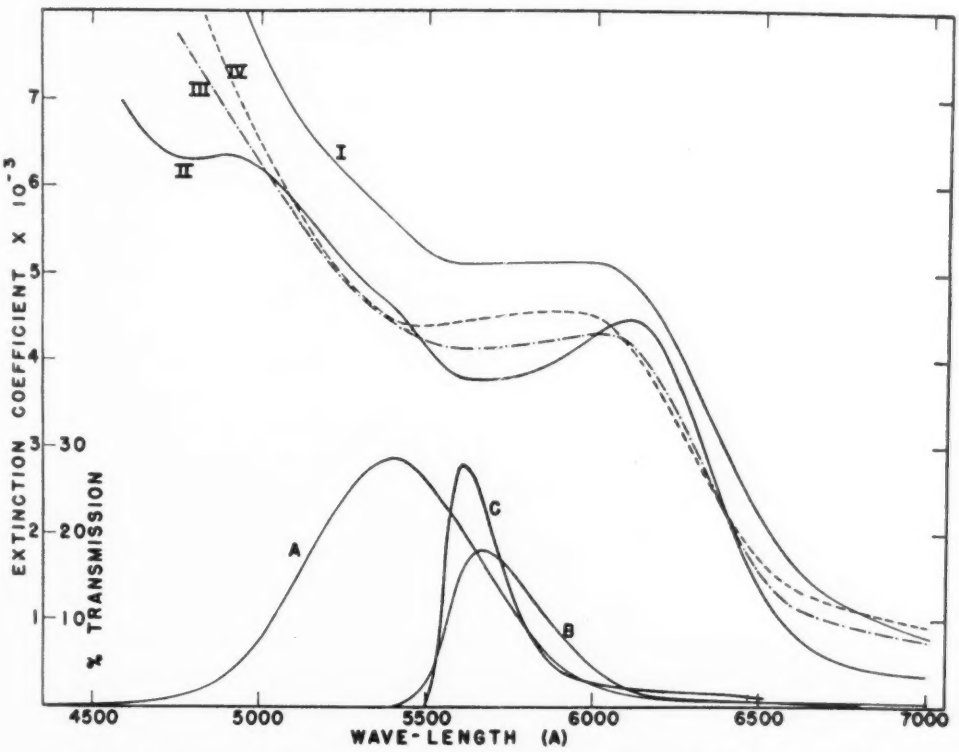


Fig. 1.—The absorption spectra of solutions of alkaline hematin. Curve *I*, alkaline hematin prepared from whole blood in N/10 NaOH; Curve *II*, crystalline hemin in N/10 NaOH, fresh solution; Curve *III*, crystalline hemin in N/10 borate buffer pH 9.4, fresh solution; Curve *IV*, crystalline hemin in N/10 borate buffer pH 9.4, diluted from aged stock solutions. Curve *B* represents the transmission in per cent for the 5,700 A filter described in the text. Curve *A* is the transmission of the Chance green filter; Curve *C* the transmission of the Evelyn 565 filter.

solutions may be used for one month. In the case of two preparations which had been kept five and eleven months, respectively, a decrease in absorption in the green was observed. Stock solutions should be discarded after they are one month old, and standards diluted from the stock solutions should be prepared fresh on the day they are required.

*Crystalline Hemin as a Primary Standard.*—The solutions of hemin in borate buffer have been applied to the analysis of blood by the alkaline hematin method, using the portable photoelectric colorimeter previously described[8] as well as the Evelyn colorimeter. Two of the portable instruments were used in the study. For the determination of alkaline hematin, a filter was selected to

give maximum transmission at approximately 5,700 A, as shown in Fig. 1, Curve B. This filter is composed of the following Corning glasses: No. 9780, 2.5 mm.; No. 3482, 2.7 mm.; and No. 5031, 1.5 mm. With this filter, both solutions of alkaline hematin, whether from blood or from crystalline hematin, follow the Lambert-Beer law. From theoretical considerations, the 5,700 A filter should be superior to the Chance green filter used by Clegg and King, Curve A, since most of the light transmitted coincides with the flat portion of the alkaline hematin spectrum. With the Evelyn colorimeter, the 565 filter, Curve C was used.

In Table II are given the results obtained from a comparison of solutions of alkaline hematin prepared from crystalline hemin with solutions of alkaline hematin prepared from blood as previously described.

TABLE II. HEMOGLOBIN EQUIVALENT OF CRYSTALLINE HEMIN IN BORATE BUFFER AT PH 9.4

| INSTRUMENT | BLOOD IN N/10 NaOH (C IN GM./100 C.C.) | HEMIN IN N/10 BORATE BUFFER pH 9.4 (C IN MG./LITER) | MG./LITER HEMIN EQUIVALENT TO 16.7 GM./100 C.C. HEMOGLOBIN |
|---|---|---|---|
| Colorimeter A (1) | $\log \frac{100}{T} = 0.0131C$ | $\log \frac{100}{T} = 0.00729C$ | 30.0 |
| Colorimeter A (2) | $\log \frac{100}{T} = 0.0121C$ | $\log \frac{100}{T} = 0.00628C$ | 30.2 |
| Colorimeter B (1) | $\log \frac{100}{T} = 0.0133C$ | $\log \frac{100}{T} = 0.00748C$ | 29.7 |
| Colorimeter B (2) | $\log \frac{100}{T} = 0.0130C$ | $\log \frac{100}{T} = 0.00746C$ | 29.1 |
| Evelyn | $\log \frac{100}{T} = 0.217C$ | $\log \frac{100}{T} = 0.01250C$ | 29.0 |
| | | Average | 29.6 |

For each colorimeter, a calibration curve was constructed by plotting the logarithm of the transmission against the concentration of hemoglobin in the blood sample. A similar plot was made for the crystalline hemin data. Each of the curves was fitted by the method of least squares. In Table II are contained the equations obtained and the values of the hemoglobin equivalent for the hemin standards, computed by simultaneous solution of the equation pairs.

The calibrations designated (1) and (2) for colorimeters A and B were separated by a period of a month, during which time changes in the amplifier circuit of colorimeter A were made. As will be noted from the corresponding equations, the transmission values were considerably different, yet there was no change in the value of the hemoglobin equivalent of the hemin standards. Since the amplifier changes resulted in a higher sensitivity, and therefore a lower lamp temperature, this observation is offered as an indication that such changes will not affect the value of the hemin standards.

From Table II it will be seen that 29.6 mg. of hemin per liter of N/10 borate buffer will, when prepared as described, give the same transmission as a blood sample containing 16.7 Gm. per 100 c.c. of hemoglobin when the blood samples are converted to alkaline hematin at a dilution of 1:251. In terms of molecular

concentration, 1.14 molecules of crystalline hemin are required to give the same absorption as will be obtained from 1.00 molecules of hemoglobin. This ratio is in excellent agreement with that observed spectrophotometrically at about 5,700 A.

Presumably because of the calcium oxalate formed, oxalated blood samples give a somewhat lower transmission than do nonoxalated samples. The results given in Table II were obtained with oxalated blood. With blood diluted directly from a finger puncture, the absorption values, and therefore the color ratio, are about 3 per cent lower.

TABLE III. COMPARISON OF COLORIMETRIC ALKALINE HEMATIN AND SPECTROPHOTOMETRIC OXYHEMOGLOBIN METHODS OF HEMOGLOBIN DETERMINATION

| BLOOD SAMPLE | SPECTROPHOTOMETRIC GM./100 C.C. | COLORIMETER A GM./100 C.C. | COLORIMETER B GM./100 C.C. | EVELYN GM./100 C.C. |
|---|---|---|---|---|
| 1 | 13.4 | 13.5 | 13.6 | 13.7 |
| 2 | 15.5 | 15.7 | 15.5 | 15.3 |
| 3 | 17.5 | 18.0 | 17.3 | 18.0 |
| 4 | 13.4 | 13.5 | 13.4 | 13.7 |
| 5 | 13.0 | 13.1 | 13.1 | 13.0 |
| 6 | 16.3 | 16.3 | 16.2 | 16.2 |

In Table III are given the results obtained by the alkaline hematin method when compared with analyses on the Beckman spectrophotometer. The samples were determined as oxyhemoglobin at four wave lengths to insure the absence of significant quantities of other forms of hemoglobin. The spectrophotometric method had previously been calibrated by Van Slyke oxygen capacity determinations[9] and found to agree within 1.0 per cent with this method. For the alkaline hematin method, the accuracy is about 0.2 Gm. per 100 c.c.

## DISCUSSION

For the purposes of photoelectric colorimetry, standard solutions must have absorption spectra closely approximating those of the unknown solutions. Only when this condition is satisfied will changes in filter transmission, in photocell sensitivity, and in lamp color temperature affect both standard and unknown in a parallel manner and permit the standard to retain its validity in the event of such changes. It is further desirable that the standard solutions be stable over long periods of time in order to obviate the necessity for frequent preparation of fresh standards. Neither of these requirements is adequately met by solutions of crystalline hemin in N/10 NaOH. As pointed out by Collier, and in the present work, these solutions are unstable and qualitatively different in spectrum from alkaline hematin prepared from whole blood. Both the stability and similarity to blood alkaline hematin are substantially improved by solution at the lower pH.

## SUMMARY

1. Standard solutions for the colorimetric determination of hemoglobin by the alkaline hematin method may be prepared from crystalline hemin in N/10 borate buffer at pH 9.4. Stock solutions containing 1 mg. per cubic centimeter must be aged overnight before dilution.

2. These standard solutions give absorption spectra similar to that given by alkaline hematin derived from blood.

3. With a yellow-green filter, 29.6 mg. crystalline hemin per liter of N/10 borate buffer at pH 9.4 will have an absorption equal to that of a blood sample containing 16.7 Gm. per 100 c.c., diluted 1:251 in N/10 sodium hydroxide. On a molecular basis, the ratio of absorption intensities is 1:1.14.

4. The accuracy of the alkaline hematin method is 0.2 Gm. per 100 c.c.

The author is indebted to Dr. E. J. King, for several specimens of the Chance green filter, to Mrs. Dorothy Peterson, for valuable assistance in the spectrophotometric measurements, and to Dr. Herman Yagoda, for the gravimetric iron determinations.

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# A SIMPLE ELECTROCARDIOGRAPH CONTACT ELECTRODE FOR USE WITH THE SMALLER LABORATORY ANIMAL

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Chicago, Ill.

EXPERIMENTAL electrocardiogram studies in relation to nutrition, as well as to other factors that will cause disturbances of cardiac function, have always had the inherent disadvantage of not having a suitable simple electrode available for use with the smaller animal. The smaller animal is usually preferable or demanded in these types of experiments for economic reasons. The usual procedure has always been to devise a suitable electrode contact for the animal to be utilized in the study at hand. This has not always been practical, since the procedure is time consuming in the period the investigator discovers what will and what will not work satisfactorily. The expense of losing what might have been valuable records should also be taken into consideration.

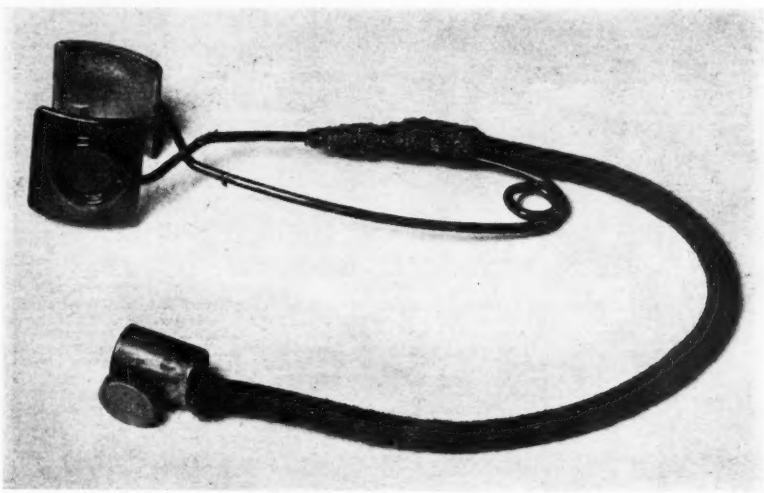

Fig. 1.—Photograph of a simple electrocardiograph electrode clip for use with smaller animals.

Illustrated in Fig. 1 is an electrode designed and constructed in this laboratory for use with the smaller unanesthetized rabbit in conjunction with dietary deficiency diseases and their effect on the heart. The electrode clip, as used with the rabbit, has a length proper of three inches, width of three-fourth inch, conventional round spring of one-half inch diameter for tension, soldered wire connection, and is insulated with a suitable material except for the inner terminal facings. Spring brass wire of 0.0575 inch diameter was used

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for constructing the clip. The tension is of the order of 125 Gm. at the electrode surface. The surface of the electrode to be attached to the animal is solid, elliptic, brass sheeting three-fourth inch long and one-half inch wide which fits snugly over the animal's leg. Materials used were of low electrical resistance. The

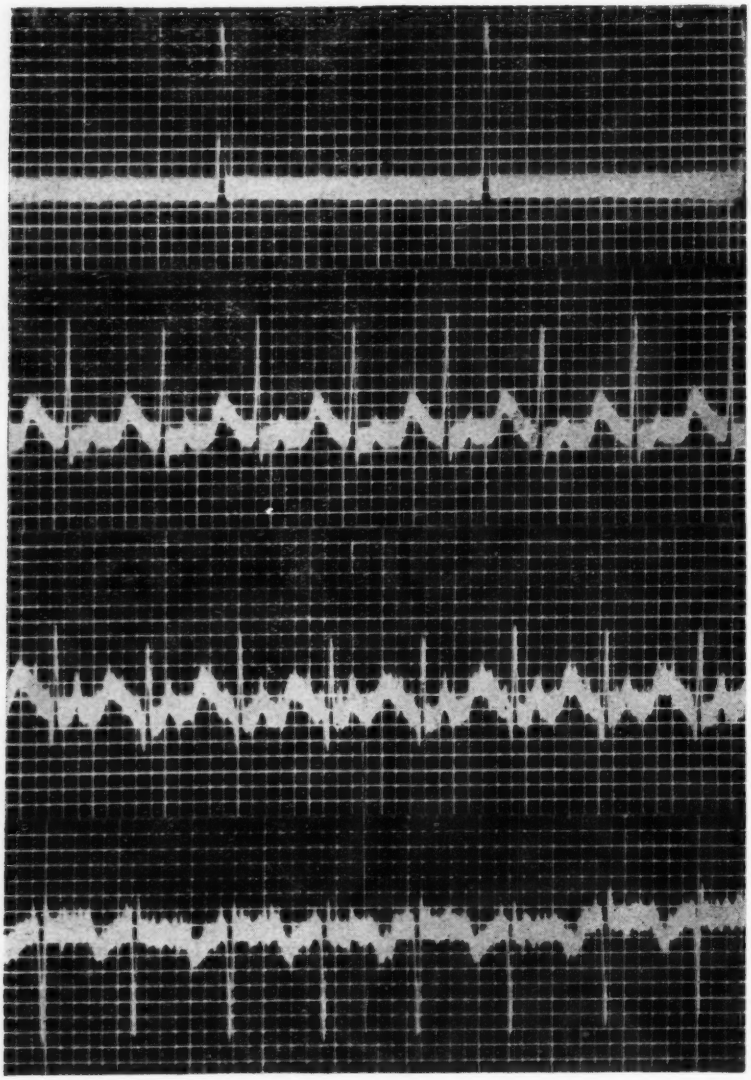


Fig. 2.—Electrocardiogram tracings of a normal rabbit weighing 1,200 grams made with the electrodes described.

length of the wire to the binding post should depend on the nature of the apparatus used to hold the animal. The design of the electrode was made of such a nature that it could be altered to be applicable for any size animal. Rabbits weighing 700 grams produced good results.

The electrode clip was designed so that the comfort of the animal could be preserved and there still be the retention of contact. It was constructed so that the pressure resulting would not stop circulation nor cause pinching of the leg and struggle from pain.

The electrocardiographic tracings of the three conventional leads of a normal rabbit, using the previously described electrode clips, are presented in Fig. 2. The animal was allowed to sit without anesthesia, and without being in a box, to prevent struggling. The clip electrodes were attached to the animal with contact being made through the use of concentrated sodium chloride as the electrolyte or by use of ECG paste. The spring held the electrode in place on the leg. If necessary, brief anesthetic quantities of pentothal sodium may be used to quiet the animal and dispel muscle action potentials without interfering with the electrocardiogram.*

The instrument used was a General Electric, Victor, Model A Electrocardiograph. The sensitivity was set at 4 on the instrument. Upward deflection represents 1 millivolt for 1 cm. displacement. The time interval, vertical lines, represent one twenty-fifth of a second per line. With this electrode clip, the ordinary electrocardiograph was found to be of use in small animal studies.

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# A NEW TYPE OF GLASS CAGE FOR METABOLISM STUDIES*

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FOR metabolism experiments with small animals, cages which provide a satisfactory method for separation of fecal matter from urine are highly desirable. Most of the cages described in the literature to the present time are modifications of one type. Probably the earliest and most satisfactory of these variations was used and described by Mitchell and Carman.‡ Cages of this type have several disadvantages resulting primarily from the use of filter paper to absorb the urine. First, between collection periods, feces and scattered feed are in contact with the moist filter paper, increasing the possibility of transfer of the substance under investigation. Second, the large area exposed by the filter paper may cause some loss or oxidative change to take place in the specific fraction of the urine. Third, absorption of the urine on filter paper necessitates subsequent extraction for recovery.

To avoid these objectionable features, we designed as a substitute the cage described in this article and have used groups of them very satisfactorily for metabolism studies with albino rats.

Referring to Fig. 1, the body of the cage was constructed from a one-gallon glass jug by cutting it off about one inch above the bottom and inserting the neck end through a nine-inch tripod on which it rests and is held in an upright position. A cover for the top of the cage was made from one-half inch mesh hardware cloth and was held in place by a suitable weight laid on top of the cover. The floor consists of a circle of this wire which was cut to fit inside of the jug and was suspended by wires from the upper edge. The height to which this false bottom is adjusted will depend upon the size of the rat and the distance to the watering tube inserted through the cover. A smaller circle of twenty-mesh nichrome wire gauze supported just above the neck of the jug formed a "tray" on which feces were collected. A rigid loop of wire attached to the tray made a convenient handle for removing the tray and its contents. The neck of the jug was fitted with a rubber stopper through which a straight glass tube was passed until it was flush with the small end of the stopper. The urine drained through this tube and was collected beneath in a 200 c.c. Erlenmeyer flask, which contained approximately 30 c.c. of 0.1 N sulfuric acid. The figure and description demonstrate the cage as used by us for a nitrogen metabolism experiment. Each day during the

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†Present address: The Grapette Co., Camden, Ark.
‡Mitchell, H. H., and Carman, G. G.: Biological Value for Maintenance and Growth of Proteins of Whole Wheat, Eggs, and Pork, J. Biol. Chem. **60**: 613, 1924.

collection period, the feces were removed and preserved under alcohol containing several drops of concentrated $H_2SO_4$. The neck and wire circles of the cage were rinsed with hot 0.1 N $H_2SO_4$ which drained into the flask. The urine in acid solution was transferred to a bottle containing several small

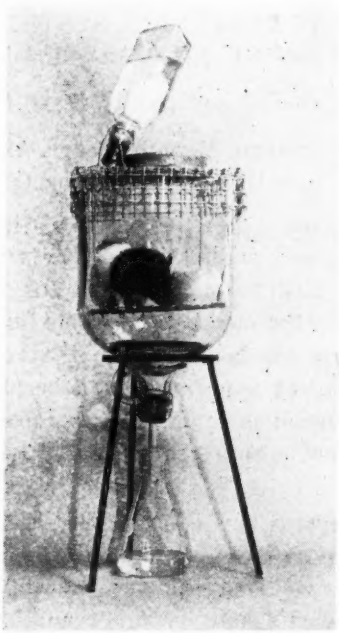

Fig. 1.—A new type of glass cage for metabolism studies.

crystals of thymol and was stored until the end of the collection period. The cage was again made habitable by flushing out the residual acid with water.

Ease of construction and efficient manipulation render these cages very desirable, especially since necessary materials are commonly found in laboratories doing biologic work with small animals.

# AN ISOLATED HEART PERFUSION SYSTEM ADAPTED TO THE DETERMINATION OF NONGASEOUS METABOLITES

## With Sample Data Upon the Isolated Monkey Heart

Captain Maynard B. Chenoweth, Medical Corps, Army of the United States, and Ethol Shiels Koelle

STUDIES of the nongaseous metabolism of the heart during the action of drugs have not often been made. When they are, heart-lung preparations of the Starling type are most frequently employed. Data obtained upon such systems inevitably include the metabolism of the lungs, which is often significant.[1] Although a system has been devised to eliminate this difficulty,[2] it is not a particularly convenient apparatus. Furthermore, such systems depend upon blood for the perfusion medium. While blood is admirably suited for many purposes, it does not permit ready manipulation of concentrations and varieties of components.

The system of continuous perfusion to be described is simple and readily constructed in most laboratories. A modified Locke-Ringer solution is employed as the perfusate, allowing variable concentration of any component. Mechanograms of the heart's activity are readily obtained and show little variation or depreciation over a period of many hours. Furthermore, errors due to bacterial metabolism are largely eliminated by taking advantage of the bactericidal properties of ultraviolet light. The results of the past year's work with this system have been so satisfactory that publication of the details of the method seems warranted.

### APPARATUS

In Fig. 1 is shown the customary setup for such work. The water bath, *5*, surrounding the weighted reservoir, *4*, is maintained at 38.5° C. and contains the outflow siphon, *1*, and oxygenating tube, *2;* a cold finger which effectively limits evaporation is represented by *3*. Not shown is the extension of *7*, the return tube, which is directed into the reservoir. The outflow tube requires an outer jacket of large rubber tubing during very cold weather but is ordinarily uninsulated. An excess length of rubber tubing is provided above the long cannula, *8*, to permit removal of the cannula to a lower and more convenient position for the actual cannulation. The cannula is held in a spring clamp for ready transfer. The perfusion pressure is arranged to be no more than 50 cm. of perfusate. Raising the pressure above this level results in rapid edema formation and early failure. Care must be exercised in placing a tuft of well-

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washed glass wool in the top portion of the cannula so this will be neither too tight to permit a free flow of perfusate nor too loose to be an effective filter. A beam of ultraviolet light, *6*, from a microscope substage lamp containing a mercury arc bulb* is focused upon the cannula and limits bacterial growth below 100 organisms per cubic centimeter after eight hours of perfusion, compared to several millions of organisms without irradiation. No special glass

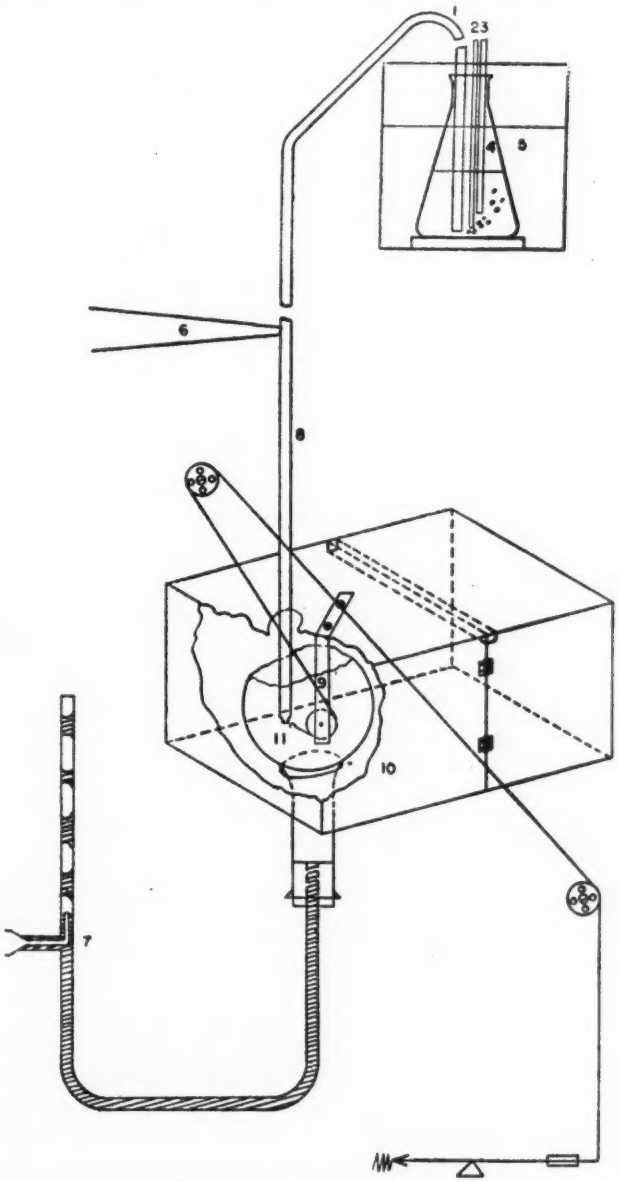
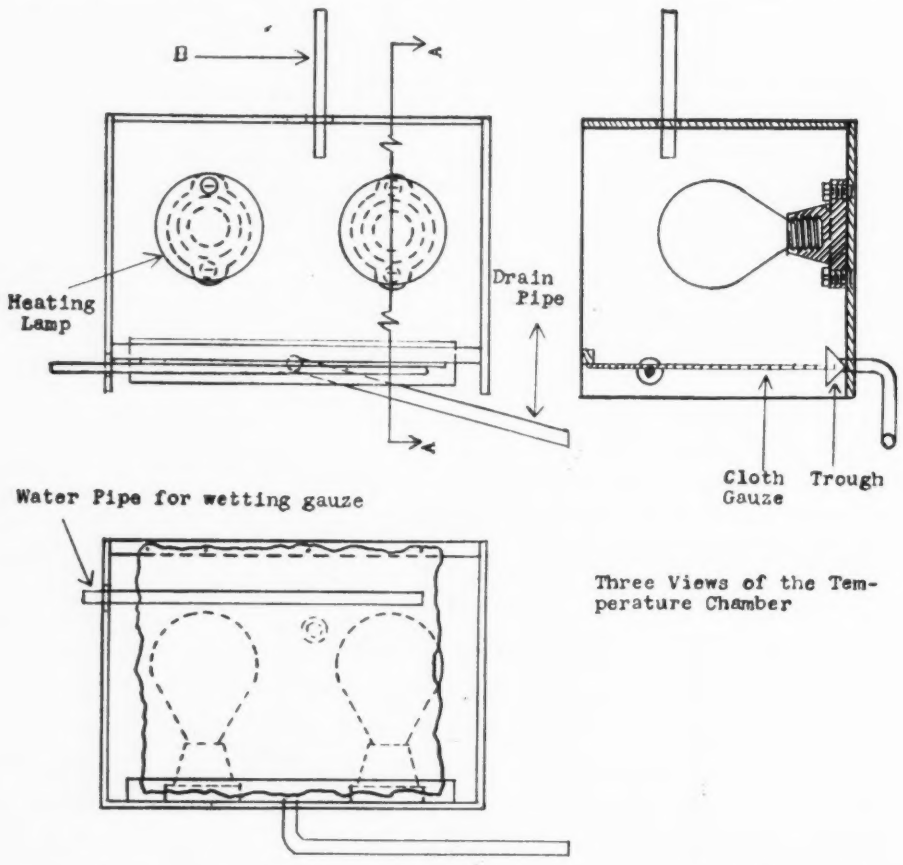



Fig. 1.—General plan view of the perfusion system. For details see text.

*General Electric Mazda AH1.

was employed for the cannula, ordinary soft glass being satisfactory. No untoward effects of the irradiation upon the experiments have been detected.

The heart chamber, *10*, is maintained at 39° C. by two 150-watt electric light bulbs and a thermostat (Fig. 2). Mixing of the air within the chamber is accomplished by the air jet shown behind the bulbs. Humidity is maintained by streams of warm water sprayed upon an evaporator formed of gauze pads suspended over a drain trough placed between the bulbs and the heart.




Three Views of the Temperature Chamber

Fig. 2.—Two views of the portion of the heart chamber occupied by the heating and humidifying elements. At *A* are connected the terminals from the thermostat system indicated in Fig. 3. An air stream is introduced at *B*. Not shown are the numerous perforations along the side of the water sprayer of the humidifier.

The heart is mounted on the cannula and well within a bottomless 150 c.c. Florence flask, *11*, inverted through a hole in the bottom of the chamber. This receptacle is protected by a cellulose acetate screen against splashing from the humidifying system. The necessity for sudden changes in humidity or temperature is minimized by the provision of a transparent plastic door on the heart chamber. Dipping down into the receptacle is a glass and plastic pulley, *9*, mounted on a flexible lead bar attached to the top of the chamber (Fig. 3).

The lead is tightly wrapped with rubber dam to exclude accidental contamination of the perfusate. The glass bearings and axle of the pulley are simply made, the former by indenting the heated end of a glass rod with a sharp pencil and the latter by slowly turning a glass rod to a point against a fine abrasive stone. An almost frictionless bearing is thus obtained. Metal pulleys may not be used in this position, as the perfusate would soon contain toxic amounts of metallic salts.

A solid column of perfusate is maintained through the return system (Fig. 1) below the receptacle to the point, 7, where the upward air jet is admitted.

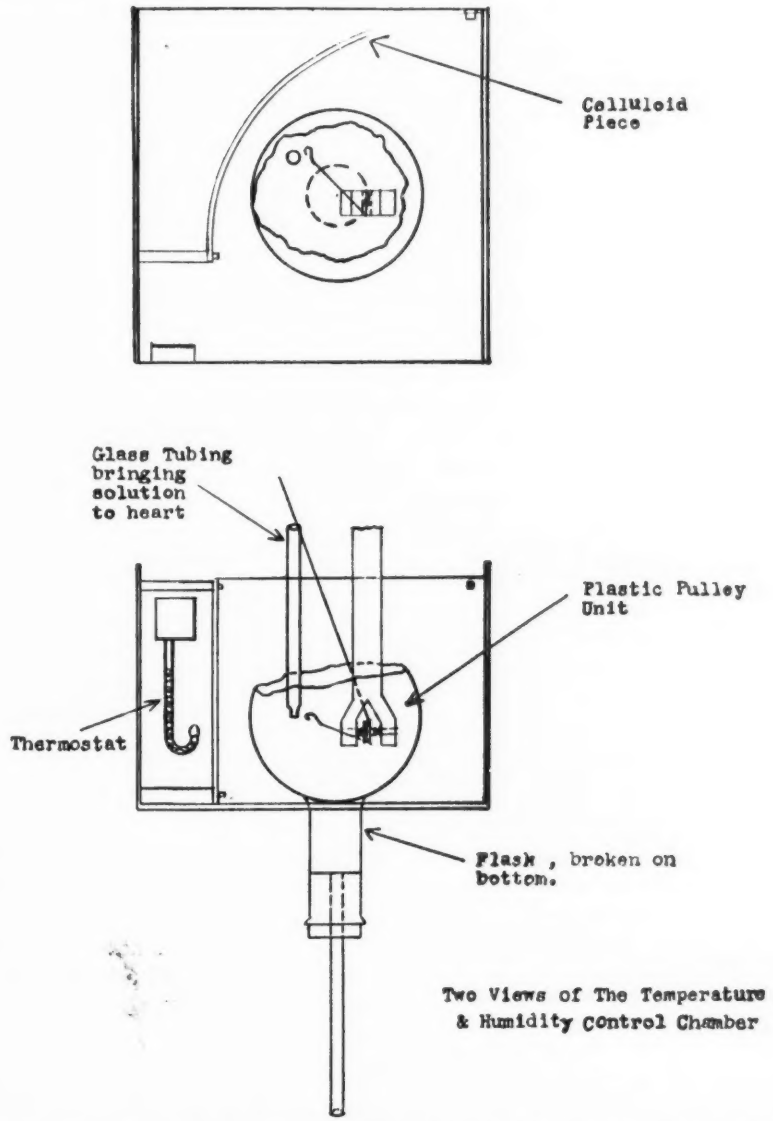

Celluloid Piece
Glass Tubing bringing solution to heart
Plastic Pulley Unit
Thermostat
Flask, broken on bottom.
Two Views of The Temperature & Humidity Control Chamber

Fig. 3.—Two views of the other half of the heart chamber (see Fig. 2). The "celluloid piece" protects the heart from splashing from the humidifier.

This simple but little-used device, introduced for this purpose by Locke and Rosenheim,[3] may be said to break off portions of the perfusate column which are then blown up to the reservoir. The hydrostatic pressure of the interrupted fluid column in the upflow tube is much less than the hydrostatic pressure of the solid column in the downflow tube. A proportion of 1 cm. of solid column in the downflow tube to 3 cm. of broken column in the upflow is easily handled by the system. Some experimentation may be necessary to establish the ideal conditions for each installation.

TABLE I. COMPOSITION OF PERFUSION FLUIDS*

| SUBSTRATE MATERIAL | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $NaHCO_2$ | SUBSTRATE |
|---|---|---|---|---|---|---|
| Glucose | 7.0 | 0.42 | 0.24 | 0.20 | 2.1 | 1.80 |
| Acetate, Na | 6.0 | 0.42 | 0.24 | 0.20 | 2.1 | 0.77 |
| Pyruvate, Na | 6.5 | 0.42 | 0.24 | 0.20 | 1.8 | 0.78 |

*All weights are grams per liter of anhydrous salts.

The modified Locke-Ringer solutions which have been successfully employed are indicated in Table I. All salts must be of the highest purity. Sodium chloride labelled "for biological work" (Merck) is a most suitable preparation. Other forms of chemically pure sodium chloride have often been found unsatisfactory. An anhydrous glucose manufactured by Baker Chemical Company is suitable, but ordinary U.S.P. glucose is not. A mixture of 95 per cent oxygen and 5 per cent carbon dioxide is used to oxygenate and to maintain the pH of the perfusate. The latter always measured from 7.3 to 7.4 (glass electrode) when the temperature of the reservoir was 38.5° C. All gases are admitted to the system after bubbling through distilled water.

Certain other precautions are imperative for the procurement of long-lived preparations. All water which is used to make up the perfusate is redistilled through an all-glass system, and the perfusion system is rinsed several times with this glass-distilled water after cleaning. All rubber tubing must be uncolored gum rubber and must be boiled for several hours in distilled water. The system must not be cleaned with chromate-sulfuric acid mixtures which leave significant amounts of highly toxic chromium salts despite diligent washing. Protein is removed from the glass portions of the system with hot nitric acid, but cork and rubber portions must be cleaned with soap or hydrochloric acid. Unrelaxed precaution must be observed against the introduction of metal into the apparatus where any possibility of contact with the perfusate exists. For example, condensate dripping off metal clamps is not always immediately detected, while iron filings have been found in the best glass wool. The hook used to attach the writing lever to the heart is made from a detempered stainless steel hypodermic needle. It may be pointed out that each precaution is based upon experience and cannot be overlooked successfully.

## PROCEDURE

In the study for which this apparatus was employed, the procedure to be described was regularly followed. The perfusate was made up in advance,

omitting the substrate and the sodium bicarbonate. Following thorough equilibration of the solution against the gas mixture, the substrate and sodium bicarbonate were added. Failure to acidify the perfusate before addition of the bicarbonate often results in the formation of a precipitate of calcium carbonate, rendering the solution useless. A measured quantity of the perfusate was added to the system and the perfusate started through the cannula and allowed to circulate slowly until temperature, and hence pH, equilibrium was reached. The ultraviolet irradiation was begun at once. A large, flat, glass dish was placed in a convenient place to catch perfusate lost while the heart was being attached to the cannula, a screw clamp being adjusted to maintain a slow rate of flow during the process. The volume thus lost was subtracted from the amount originally in the system. The animal was killed by crushing the head, chemical anesthesia of any form having been found severely depressant to hearts obtained from such animals. The heart was quickly removed and the aorta cannulated after trimming and washing. All clots were removed by forceps and washing before mounting. Hearts which develop ventricular fibrillation during this process should be discarded even though arrest of the fibrillation is prompt, as experience has shown their inferiority. An effort was made to tie below the innominate artery without occluding the pulmonary artery, which must otherwise be cut open, occasionally inducing ventricular fibrillation. The clamp was then opened fully, the heart flushed with from 50 to 100 c.c. of fluid, the clamp tightened, and the cannula and heart were mounted in the heart chamber. The clamp was released immediately afterward. The heart lever system was securely attached to the heart and was arranged to record on a slowly moving kymograph.

Preparations thus obtained were often found to manifest little or no depreciation before nine or ten hours of perfusion, monkey hearts being more durable than rabbit hearts. Samples for analysis were removed by a syringe and a hypodermic needle from the rubber tubing above the cannula. The amount of concentration resulting from evaporation of the perfusate was corrected from $Cl^-$ levels. Drugs may be added to the system through the top of the receptacle flask, permitting solution and distribution before entering the heart. The circulation time for added dyes was about fifty seconds in the apparatus employed.

## RESULTS

In Fig. 4 are shown kymographic tracings of three control experiments on monkey hearts *(Macaca mulatta),* with a perfusate containing 0.01 M glucose. The preparations were allowed to beat for six hours, with the noteworthy minimum of failure shown. Analytic results obtained on these three hearts are presented in Table II.* The slight degree of failure (11 per cent overall) noted in Experiment 104M7 is closely paralleled by a sharp increase in the

*Glucose was determined by the method of Somogyi,[4] pyruvic acid by the method of Friedemann and Haugen,[5] lactic acid by the method of Barker and Summerson,[6] and keto acids by the method of Friedemann and Haugen.[5]

production of lactic and pyruvic acids, absent in the other two experiments. The increased disappearance of glucose during the second three-hour period noted in the first two experiments is generally observed and may be interpreted as the result of the disappearance of cardiac glycogen, hearts beating in substrate-free perfusate customarily failing at the end of a three-hour period, indicating that cardiac glycogen has been exhausted. Therefore, these

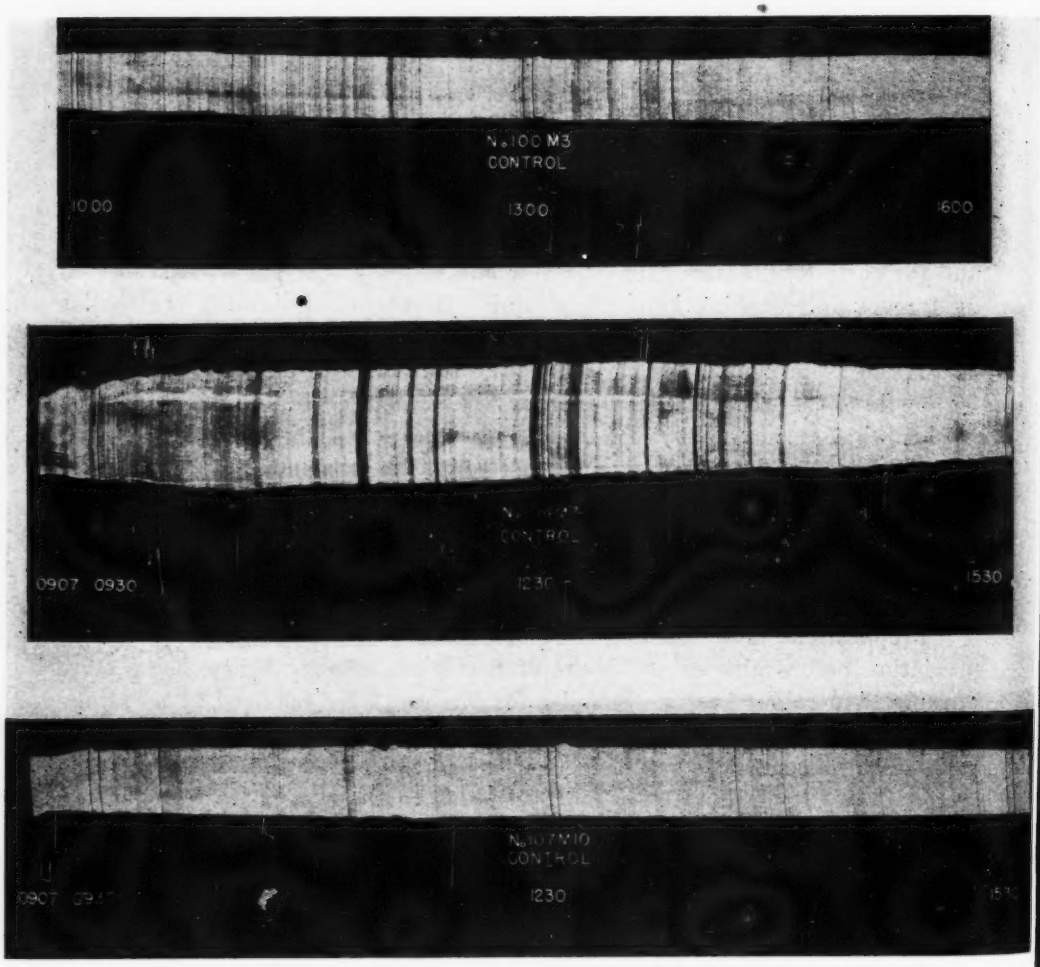



Fig. 4.—Three kymographic tracings of isolated, perfused monkey hearts (*Macaca mulatta*). Substrate 0.01 M glucose. Time is Army-Navy twenty-four hour system and represents a total perfusion time between the first and third analyses of six hours.

higher figures probably represent the actual glucose utilization of glycogen-low hearts. The absence of such a change in Experiment 107M10 is taken to imply a larger reservoir of cardiac glycogen. A sample calculation for glucose utilization (or for any other metabolite) is presented, showing corrections for evaporation and sample volume.

TABLE II. ANALYTIC DATA OBTAINED ON MONKEY HEARTS USING GLUCOSE AS SUBSTRATE IN A SERIES OF CONTROL EXPERIMENTS

| EXPERIMENT | PERIOD (3-HR. INTERVALS) | CIRCULATING LEVELS MG./DL. | | | | | UTILIZATION MG./GM. DRY WT./HR. | | | | PER CENT OF INITIAL CONTRACTION |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CHLORIDE | GLUCOSE | LACTIC ACID | PYRUVIC ACID | TOTAL KETO-ACIDS | GLUCOSE | LACTIC ACID | PYRUVIC ACID | TOTAL KETO-ACID | |
| 100M3 | 0 | 910 | 115 | 0.80 | 0.30 | 0.33 | | | | | 100 |
| | 3 | 913 | 105 | 0.70 | 0.24 | 0.20 | − 5.80 | −0.04 | −0.03 | −0.07 | 103 |
| | 6 | 929 | 60 | 2.30 | 0.48 | 0.56 | −21.00 | −0.80 | +0.10 | +0.15 | 109 |
| 104M7 | 0 | 906 | 169 | 1.00 | 0.30 | 0.33 | | | | | 100 |
| | 3 | 910 | 168 | 0.50 | 0.22 | 0.17 | − 1.40 | −0.44 | −0.07 | −0.14 | 118 |
| | 6 | 929 | 133 | 3.10 | 0.54 | 0.56 | −26.70 | +1.80 | +0.22 | +0.27 | 89 |
| 107M10 | 0 | 914 | 173 | 0.60 | 0.16 | 0.15 | | | | | 100 |
| | 3 | 920 | 172 | 0.90 | 0.24 | 0.20 | − 2.50 | +0.35 | +0.10 | +0.06 | 108 |
| | 6 | 931 | 172 | 1.40 | 0.32 | 0.28 | − 1.70 | +0.47 | +0.07 | +0.08 | 157 |

*Experiment 104M7*

| | | TIME (HR.) | MG. PER CENT (OR PER DL.) GLUCOSE | CHLORIDE |
|---|---|---|---|---|
| Initial volume | = 700 c.c. | 0 | 169 | 906 |
| Initial loss | = −50 c.c. | 3 | 168 | 910 |
| | 650 c.c. | | | |

Heart weight after 24 hr. at 104° C. = 2.10 Gm.
Sample volume = 100 c.c.

*Period I*

(a) 6.50 dl. × 169 mg./dl. = 1098 mg. total
−1.00 dl. × 169 mg./dl. = −169 mg. total
5.50 remain after sample 929 mg. total

(b) Correction for evaporation:
$\frac{906 \text{ mg. Cl/dl.}}{910 \text{ mg. Cl/dl.}} \times 5.50 \text{ dl.} = 5.48 \text{ dl.}$
5.48 dl. × 168 mg./dl. = 920 mg. total
9 mg. used

(c) 9 mg./2.10 Gm. dry wt./3 hr.
or − 1.4 mg./Gm. /hr.

*Period II*

Calculations repeated commencing with the corrected 3 hr. volume, 5.48 dl.

## DISCUSSION

No data appear to be available in the literature on the behavior of isolated, perfused monkey hearts, so that no direct comparison with the data presented is possible. The data obtained, however, fall well in line with the more detailed studies on other species and have the advantage of being free from errors introduced by bacterial metabolism. The ease with which the system presented here can be routinized and adapted to many studies commends its frequent application.

## SUMMARY

1. A convenient, simple, and highly satisfactory continuous perfusion system for the preparation of vigorous, long-lived, isolated hearts has been described. The system is particularly adapted for chemical analyses under bacteria-free conditions.

2. Data are presented for three experiments on the isolated, perfused monkey heart *(Macaca mulatta)*, demonstrating the suitability of the method.

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